

Optimization of Non-Viral Transfection Methods
for Equine Mesenchymal Stem Cells

Honors Thesis
Presented to the College of Arts and Sciences
Cornell University
in Partial Fulfillment of the Requirements for the
Biological Sciences Honors Program

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May 2013

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Abstract

Mesenchymal stem cells (MSCs) have exciting medical potential, especially those that are genetically modified. Non-viral methods for genetic modification present several advantages over viral methods, and hence, such methods have been optimized for MSCs from a variety of species. Although equine MSCs have great importance, prior to this study, effective non-viral transfection parameters for these cells had not been determined. Here both chemical and physical transfection methods were optimized for equine MSCs, using red fluorescent protein as a reporter gene. Chemical reagents were optimized for reagent-to-DNA ratio, transfection solution plating volume, and cell density. Additionally, ideal voltage, cell concentration, DNA concentration, buffer, and pulse number settings were discovered for electroporation. The method resulting in highest gene expression was electroporation with one 30-msec pulse at 170 volts in Opti-Mem buffer. This technique resulted in transfection of 54% of the cell population, a percentage that is on the higher end of nonviral MSC transfection efficiencies. The transfection parameters determined in this study will undoubtedly be useful to all researchers who wish to genetically modify equine MSCs.

Keywords: cationic lipid, electroporation, equine, gene therapy, mesenchymal stem cells, nonviral gene transfer, optimize, transfection

Introduction

Mesenchymal stem cells (MSCs) have exciting medical potential, because they can be readily isolated, quickly expanded, and induced to differentiate along diverse pathways. MSCs have been obtained from many tissues, including bone marrow, skeletal muscle, pancreas, adipose tissue, synovium, primary teeth, and brain, and can differentiate into adipocytes, osteocytes, myocytes, chondrocytes, neurons, and hepatocytes^{1, 2}. They have been used experimentally to treat a number of conditions, from spinal cord, cartilage, and tendon injuries, to coronary artery disease, muscular dystrophy, lung damage, stroke, Parkinson's Disease, and anemia³. MSCs are identified according to their adherence to plastic under standard culture conditions, their expression of CD105, CD73 and CD90, their lack of expression of CD45, CD34, CD14 or CD11b, CD79-alpha or CD19 and HLA-DR surface molecules, as well as by their differentiation capabilities⁴.

Many medical applications of MSCs involve genetic modification. For instance, MSCs overexpressing CXC chemokine receptor 4 migrated towards the infarcted region of rat hearts more than unmodified MSCs, and helped in the myocardial repair process⁵. MSCs expressing tumor necrosis factor apoptosis ligand (TRAIL) had anti-cancer effects in malignant human glioma models⁶. Arthritic mice treated with interleukin-10-transduced MSCs had significantly decreased arthritis severity compared to mice treated with MSCs alone⁷. These and many other studies demonstrate the wide-ranging possibilities for medical use of modified MSCs.

The introduction of foreign DNA into MSCs can be achieved through viral or non-viral methods. Viral transduction is no doubt effective, and can result in close to 100% gene expression under certain conditions⁸. But the use of viruses as gene delivery vehicles has disadvantages, such as the high risk of an immune reaction against the virally-modified cells, or

the potential for random integration of the transgene into the patient's genome, which could lead to harmful or lethal mutations⁹. In a famous and tragic incident, 18-year-old Jesse Gelsinger died after an injection of 2.8×10^{13} adenovirus particles into his liver. These viruses carried a gene meant to correct his ornithine transcarbamylase deficiency¹⁰. Since then, researchers have made substantial progress in developing viral vectors and delivery methods that are better suited to gene therapy applications, including advances to prevent immune responses from lentiviral, adenoviral, and adeno-associated virus-mediated gene transfer¹¹. However, another promising option is to simply avoid the use of viral methods altogether. Non-viral transfection techniques have several advantages, including lower immunogenicity, lower toxicity, and less restriction on size of construct to be transferred¹².

Because of these advantages, a vast number of non-viral techniques for introducing DNA into cells have been developed. Two of the most common methods are lipofection, in which cationic lipids surround the DNA and facilitate its entrance into the cell, and electroporation, in which an electric field induces formation of pores in the plasma membrane, through which DNA can enter^{13,14}. A less-common physical method is sonoporation, in which ultrasonic waves are used to disrupt the cell membrane and allow entry of DNA into the cell^{15,16}. Recently, electroporation and sonoporation have been used together, in a technique logically termed electrosonoporation¹⁷⁻¹⁹. And there are a multitude of other methods, including use of cationic polymers, high-pressure gene delivery, particle bombardment, hydroporation, magnetofection, laser beam gene transduction, jet injection, photochemical internalization, and nanoparticle-facilitated transfection^{9, 20}.

Each of these methods must be optimized for the cell type and species of interest. Since MSCs have so many applications, researchers have already optimized different transfection

methods for MSCs derived from a number of different organisms, and it is clear that MSCs from different species have different optimal transfection parameters²¹. Table 1 provides an overview of the results of a number of optimization studies for MSCs in different species. The variability in peak efficiency for the same methods, or even the same reagents, is a reflection of species differences as well as methodological differences. Generally, physical methods perform better than chemical methods, with chemical transfection efficiencies ranging from 0 – 35% and electroporation efficiencies ranging from 2 – 90%. Nucleofection (Amaxa) is simply a trademarked variation on electroporation that uses cell-type-specific reagents.

Table 1. Efficiency of Non-Viral Transfection Methods for MSCs. Presented here are results from a variety of previous studies that optimized one or more nonviral transfection methods for MSCs. If a percentage of expressing cells was not reported, “n.r.” is listed under Peak Efficiency. Only the transfection efficiency resulting from the optimized set of parameters is listed under Peak Efficiency.

Study	Organism	Type of method	Specific method	Peak Efficiency (%)
Hamm et al. ²²	Human	Physical	Nucleofection	45
		Chemical	Chemical: Lipofectamine PLUS, DOTAP, Effectene, Superfect, BES	0
Haleem-Smith et al. ²³	Human	Physical	Nucleofection	90
Aluigi et al. ²⁴	Human	Physical	Nucleofection	27
		Chemical	FuGene6 DOTAP	3.6 5.4
Helledie et al. ²⁵	Human	Chemical	Lipofectamine 2000, Lipofectin, FuGene5, Lipofectamine PLUS, Transfectin	15, 10, 10, 5, 5
		Physical	Electroporation	90
Madiera et al. ²⁶	Human	Chemical	Lipofectamine 2000	35
Lim et al. ²⁷	Human	Physical	Microporation (a variation on electroporation)	83
		Physical	Electroporation	30 – 40
		Chemical	Lipofection	< 20
McMahon et al. ²⁸	Rabbit	Chemical	Lipofectin	24
Song et al. ²⁹	Chick	Physical	Molecular vibration induced by an electric field (no electric current passes through the cell solution; transfection is mediated by the vibrations themselves)	74
Stiehler et al. ¹²	Pig	Various	Cationic liposomes, electroporation, magnetofection, calcium phosphate co-precipitation	< 2
Gheisari et al. ³⁰	Rat	Chemical	Effectene, FuGENE HD, Lipofectamine 2000, Polyfect, Superfect	8.7, 5.2, 19.6, 16.3, 9.6
Ferreira et al. ³¹	Rat	Physical	Electroporation	29
Chen et al. ³²	Rat	Chemical	Plasmid-encapsulated polyethylene glycolsylated polyethylenimine nanoparticles	15 – 21
Cao et al. ³³	Rat	Chemical	Calcium phosphate nanocomposite particles surrounding plasmids	n.r. (“similar to Lipofectamine 2000”)
Deng ³⁴	Rat	Chemical	Ethylenediamine-modified polysaccharide from mulberry leaves	n.r. (“slightly higher than Lipofectamine 2000; higher than PEI”)
Santos ³⁵	Rat	Chemical	Receptor-mediated delivery using PAMAM dendrimers conjugated with peptides recognized by MSCs	n.r.

Surprisingly, none of these studies focus on optimizing transfection of equine MSCs. Equine MSCs are of interest both for their direct clinical applications to horse health, as well as for understanding possible human disease treatments. Horses are excellent model systems for study of human chondral repair, which can be mediated by use of MSCs. Not only does horse articular cartilage have limited healing capability, as in humans, but horses are also large enough to approximate lesion size found in humans—something that cannot be done in smaller models such as the mouse. Horses have similar knee anatomy to humans, and suffer from many of the same conditions, such as various cartilage injuries and osteoarthritis³⁶. Genetically modified equine MSCs can be used to treat some of these conditions. For instance, MSCs engineered to express insulin-like growth factor-1 were used to improve healing of lesions in equine tendons³⁷.

Because of the many important applications of equine MSCs, and the current absence of studies that optimize their transfection, this study focused on discovering the best parameters for non-viral DNA delivery to equine MSCs.

Materials and Methods

Plasmid Preparation

Transgenic *Escherichia coli* carrying the pCMV-tdTomato plasmid were cultured overnight at 37°C in Luria-Bertani (LB) broth with kanamycin (50 mg/L). pCMV-tdTomato is a 5392 bp plasmid that contains a CMV-driven expression cassette for the red fluorescent protein gene tdTomato (Clontech), as well as a kanamycin/neomycin resistance gene. Plasmid was purified using a commercially available plasmid preparation kit, according to the manufacturer's instructions (Quiagen HiSpeed Midi/Maxi Plasmid Purification Kit, Invitrogen PureLink HiPure Plasmid DNA Megaprep kit). Plasmid purity was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). pAAV-eGFP plasmid was also prepared in this manner. pAAV-eGFP is a 5919bp plasmid that carries an enhanced GFP expression cassette between inverted terminal repeats, as well as an ampicillin resistance gene, driven by a CMV promoter.

MSC Isolation, Freezing, and Thawing

All tissue culture protocols were carried out in a laminar flow hood using sterile techniques. MSCs were isolated from bone marrow of a healthy horse using either a 24-hour split method or a no-split method (Appendix 3). These isolation methods, which are based on differential adherence to plastic and other specific culture conditions, have been previously determined in this laboratory to result in purified populations of MSCs from a variety of horses. The cells from those horses were verified as MSCs according to their differentiation into adipocytes, chondrocytes, and osteocytes. Because the proper isolation procedures were followed here, and the procedures have been shown to produce populations of MSCs, it was reasoned that the cells isolated for these optimizations were MSCs.

After isolation, MSCs at passage 2 were suspended in MSC Freeze Media (all media formulations in Appendix 2) at a concentration of 5 million cells per ml. One-milliliter aliquots of this solution were placed in cryovials (Thermo Scientific Nalgene Cryogenic Vials, Rochester, NY) and gradually brought to -80°C by being placed in a Styrofoam box in a freezer overnight. Subsequently the cryovials were transferred to a liquid nitrogen tank for long-term storage at -196°C (Thermo Scientific Locator 8 Cryotank).

Upon removal from storage, the vials of MSCs were placed in a water bath at 37°C until thawed. The thawed cell suspensions were immediately transferred to a 50 mL centrifuge tube containing at least 5 mL of 10% fetal bovine serum MSC media (Appendix 2) for each cryovial, or at least 20 – 30 mL of media. MSCs were pelleted by centrifugation for 5 minutes at $300 \times g$ in a swinging bucket rotor. The supernatant was removed from the pellet, and the cells resuspended in 10% or 20% FBS MSC media. After counting, cells were plated at the desired density on tissue culture plates (Greiner Bio-One or Corning Costar). MSCs were incubated at 37°C , 90% relative humidity, and 5% CO_2 (Thermo Scientific Forma Steri-Cult 200 CO_2 Incubator).

MSC Passaging

To passage cells, the media was aspirated from the culture vessel and the monolayer rinsed with Hank's Balanced Salt Solution (HBSS) (HyClone Thermo Scientific). An appropriate amount of 1X trypsin EDTA was added to cover the monolayer (0.25% Trypsin/2.21 mM EDTA in HBSS without sodium bicarbonate, Ca, or Mg. Mediatech, Inc., Manassas, VA). MSCs were incubated with the trypsin for no more than five minutes, until the cells detached. The trypsin was quenched with MSC media and the solution transferred into 50 mL conical tubes.

Centrifugation at 300 x g for 5 minutes pelleted the cells, after which they were counted and plated at the desired density. Because MSCs begin to show changed expression of surface markers and unusual morphologies beyond passage 7, only MSCs at passage 5 or earlier were used in this study³⁸.

Fluorescence-Activated Cell Sorting: Overview

Fluorescence-activated cell sorting (FACS) was employed to compare the expression of RFP and GFP by MSCs, to determine which fluorescent protein would be a better indicator of transfection efficiency—in other words, to determine whether MSCs express RFP or GFP more effectively. Additionally, this experiment was used to check whether FACS could produce a healthy, purified population of transfected MSCs.

In a second FACS experiment, cell sorting was used to verify that plate reader fluorescence data accurately indicated the transfection efficiency. Plate readings were taken on populations of cells that had been transfected with varying volumes of transfection solution, so that each population would express the transgene to a different extent. Immediately after plate reading, the MSCs were sorted by FACS. The percentage of fluorescent cells in each population was quantified by the cell sorter. Subsequently, the relationship between the plate reader fluorescence reading and the percentage of fluorescent cells was determined.

Fluorescence-Activated Cell Sorting: RFP vs. GFP Expression and Post-FACS Morphology

For determining difference in expression of GFP and RFP, cryopreserved MSCs at passage 2 were thawed and cultured on 175 cm² tissue culture plates (Grenier BioOne) at a density of 36,600 cells/cm² in 10% FBS MSC media for 48 hours. MSCs were passaged into 75

cm² plates at a density of 33,000 cells/cm² in 18 ml of 10% FBS MSC media per plate, incubated for 24 hours, and then transfected with XtremeGene HD (Roche).

Three different diluted DNA solutions (0.01 µg plasmid per µl Opti-Mem) were prepared: the first contained only RFP-encoding plasmid, the second contained only GFP-encoding plasmid, and the third contained equal concentrations of RFP-encoding plasmid and GFP-encoding plasmid (Opti-Mem + GlutaMax Reduced Serum Medium 1X, Life Technologies). These DNA solutions were used to prepare transfection complexes with XtremeGene reagent according to the manufacturer's protocol, with an incubation time of 15 minutes. A ratio of 1 µl reagent to 1 µg DNA was used, and 2800 µl of transfection solution added to each 75-cm² plate.

Prior to transfection the culture plates were rinsed with HBSS and 30 ml of 10% FBS MSC media were added to each one. The culture plates for MSCs to be transfected received 10% FBS antibiotic-free MSC media (Appendix 2), while all other plates received standard 10% FBS MSC media, which does contain antibiotics. The DNA/reagent complexes were added to the appropriate plates and the MSCs incubated with the transfection complexes for 48 hours. Some cell populations were transfected simultaneously with RFP-encoding plasmid and GFP-encoding plasmid, in order to have the most direct comparison possible between the two plasmids—to see whether a cell population presented with both plasmids in equal quantities would express one of the plasmids more effectively than it would express the other plasmid.

FACS collection tubes were coated in bovine serum albumin, to increase viability of cells after sorting. A solution of 4% BSA in 1X Dulbecco's Phosphate-Buffered Saline (DPBS) was prepared and filter-sterilized (Cellgro Mediatech Inc., Manassas, VA). The 12 x 75 mm sterile polypropylene collection tubes were filled completely with 4% BSA and incubated for at least an hour (Fisherbrand Culture Test Tube Cat. No. 14-956-1D). Polypropylene is a useful material for

collection tubes since it does not accumulate charge as much as polystyrene. Prior to sorting, the 4% BSA was poured out, and 0.5 – 1.0 ml of collection buffer was added to each tube (collection buffer: 50 ml fetal bovine serum, 0.5 ml Pen/Strep, 1.28 ml 1M HEPES buffer, sterile filtered).

FACS cell samples to be sorted were prepared in a sterile hood. In the RFP vs. GFP expression comparison test, the media from each plate was saved before the MSCs were detached using trypsin. The trypsin on each plate was then quenched using the media that had been saved from that plate. Cells were pelleted by centrifugation at 300 x g for 5 minutes. The supernatant was aspirated off and cells were resuspended in PBS (Mg/Ca⁺⁺ free) for counting. Use of Mg/Ca⁺⁺ free PBS helps prevent cells from adhering to each other, and facilitates sorting. After counting using a hemocytometer, the MSCs were re-pelleted by centrifugation, and then suspended in FACS Sorting Buffer at a concentration of 10⁷ cells/ml (FACS Sorting Buffer without DAPI: Appendix 2). Finally, the concentrated cell solutions were filtered through the caps of BD Falcon Cell-Strainer Cap 12 x 75 mm tubes (REF 352235) to reduce clumping. Prior to sorting the cell solutions were vortexed periodically, also to minimize clumping.

Samples were sorted on a FACS Aria (BD Biosciences). Dead cells were excluded on the basis of side scatter properties. After sorting, cells were plated at high density into wells of an appropriate size for the cell population, and observed at 0, 16, and 48 hours. At 48 hours, the growth media was removed and cells covered in DPBS for imaging. Cells were photographed using a Cooke SensiCam High Performance Camera (Germany) and Zeiss Axiovert S100 phase-contrast and fluorescence microscope. Subsequently only the sorted purified RFP population and non-sorted RFP population continued to be cultured. These cells were photographed again at 96 hours and then discarded.

FACS: Plate Reading Relationship to Percentage of Fluorescent Cells

In order to determine whether the plate reader output was actually an indicator of the percentage of fluorescent cells, MSCs were cultured in 96-well plates, and each plate was transfected with a different transfection solution volume so that different plates would have different percentages of fluorescent cells. The plates were analyzed with the plate reader, and subsequently analyzed by FACS, which provided the percentage of fluorescent cells. Then the plate reader output was directly compared to the transfection percentage.

MSCs were removed from cryopreservation, cultured for 48 hours on T-175s in 30 ml of 20% FBS MSC media per plate, and then passaged onto 96-well plates with 18,000 cells in 100 μ l of 10% FBS MSC media per well. Twenty-four hours later, the media was removed, each well washed with HBSS, and 100 μ l of 10% FBS antibiotic-free MSC media were added to each well. RFP plasmid DNA was diluted in Opti-Mem to 1 μ g/100 μ l, and XtremeGene transfection complexes were prepared according to the manufacturer's instructions, in a 1:2 ratio of microliters XtremeGene to micrograms DNA. Each plate was treated with a specific volume of transfection complex solution per well: 0 μ l, 16 μ l, 24 μ l, 28 μ l, or 32 μ l. Different volumes were used in order to obtain different percentages of cells expressing the gene.

After 48 hours the media and transfection complexes were removed, the wells rinsed with 1X DPBS to remove traces of media, and 100 μ l of 1X DPBS added to each well. It was important that all media be removed because the fluorescence of phenol red occurs in the same range as the fluorescence of RFP. The plates were read using a Tecan Safire Plate Reader (Austria) with Magellan V3.11 software. Subsequently, the MSCs from each plate were transferred into a corresponding centrifuge tube and pelleted. Due to the small size of the pellets, the MSCs were not counted, but rather each pellet resuspended immediately in FACS Sorting

Buffer (FACS Sorting Buffer with DAPI: Appendix 2). Samples were analyzed on a FACS Aria (BD Biosciences). Dead cells were excluded according to the degree of DAPI fluorescence.

Chemical Transfection: Overview

Three commercially available transfection reagents were tested: XtremeGene HP (Roche), FuGene HD (Promega), and Lipofectamine LTX with PLUS reagent (Invitrogen). Each reagent can be used with varying reagent-to-DNA ratios, and with varying volumes of transfection complex added to the media. These two parameters, ratio and volume, must be optimized to attain the highest possible transfection efficiency. Because of the relationship between ratio and volume—i.e. solutions with higher reagent-to-DNA ratios often have more effective transfections at lower volumes—it is imperative that each ratio be examined across all volumes, rather than attempting to optimize ratio for one arbitrarily-chosen volume or optimize volume for an arbitrarily-chosen ratio.

Once a reasonable range of values for ratios and volumes is determined, through an initial wide screen across all ratios and volumes, this range can be narrowed by further testing with more replicates of only the top ratios and volumes. This general approach was used to optimize chemical transfection: after Optimization I, in which all ratio/volume combinations for all reagents were screened, Optimization II was performed, in which only the top ratio/volume combinations for each reagent were examined in higher detail.

There are a number of options for quantifying transfection efficiency. Initially, Taqman-RT PCR on RNA was used, but this was not efficient for screening many ratio/volume combinations. Hence, a more high-throughput method was developed, to enable accurate and rapid evaluation of transfection efficiency. Cells were transfected with an RFP-encoding

plasmid, and the resulting fluorescence of the cell population quantified by a plate reader—an approach that was suggested and validated by the initial FACS experiments. The details of the protocol, including what range of ratios/volumes to test, how to calibrate the plate reader, the importance of avoiding aspiration, the timing of the transfection process, and so on, were determined through many earlier experiments that are not explicitly described here. Only the final protocol that was ultimately used to optimize chemical transfection is discussed.

Chemical Transfection Optimizations I & II: Optimization of Reagent, Ratio, and Volume

Chemical Transfection: Optimization I

This initial optimization screened both XtremeGene and FuGene across all reasonable volumes across all reasonable ratios, such that peak fluorescence readings within ratios and across ratios could be measured. Due to the high number of unique condition sets being tested, each set of conditions was used on three wells.

Cryopreserved MSCs were thawed and cultured. Cells at passage 4 were plated onto 96-well plates at 18,000 cells per well, with 100 μ l of 10% FBS MSC media in each well. After 24 hours, the cells were put into antibiotic-free media (100 μ l/well) and treated with the appropriate transfection conditions. FuGene reagent-to-DNA ratios of 3:2, 4:2, 5:2, 6:2, 7:2, and 8:2 were each tested at the following transfection solution volumes: 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, and 30 μ l added per well. XtremeGene reagent-to-DNA ratios of 1:4, 1:2, 1:1, 2:1, 3:1, and 4:1 were each tested at the following transfection solution volumes: 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, and 44 μ l added per well. Each unique FuGene or XtremeGene ratio/volume combination was tested on three wells. Lipofectamine LTX & PLUS reagent-to-DNA ratios of 1:1, 2:1, 3:1, 4:1, 5:1, and 6:1 were tested at 10, 20, 30, and 40 μ l of transfection solution added

per well, with each unique ratio/volume combination tested on 18 wells. Each plate also included cells-only, reagent-only, and DNA-only controls.

The transfection solutions were prepared according to the manufacturers' instructions, with one difference. The manufacturers recommend adjusting the initial amount of media in each well so that the final volume will be 100 μ l after the transfection solution has been added. However, instead of doing this, each well received 100 μ l of 10% FBS antibiotic-free MSC media initially, to which the transfection solution was then added. Also, extra care was taken to pipette the reagent directly into the DNA solution without contacting the sides of the tube; this minimizes the amount of reagent that adheres to the tube instead of interacting with the DNA. In preparing the FuGene transfection solution, the DNA concentration was 2 μ g of DNA per 100 μ l of Opti-Mem media, to which the reagent was added. For XtremeGene and Lipofectamine LTX, the DNA concentration was 1 μ g of DNA per 100 μ l of Opti-Mem media, to which the reagent was added.

After the appropriate incubation times (15 minutes for XtremeGene and FuGene; 10 minutes after adding PLUS and 30 after adding Lipofectamine), during which transfection complexes assemble, each unique reagent/ratio/volume combination was applied to the appropriate wells. The manufacturer warns that excessive transfection complex incubation time can adversely affect transfection efficiency, so the layout of the 96-well plates was carefully designed, and the timing at which the reagent added to the DNA solution was controlled, so as to not exceed the recommended incubation time.

After 48 hours, during which the MSCs were incubated with the transfection complexes, the media was aspirated off and plates rinsed with HBSS. One-hundred μ l of DPBS were added to each well, and the plate covered by a transparent sticker. The plate reader was used to quantify

the fluorescence in each well, with nine different reads taken in a 3 x 3 square at the bottom of each well. The gain was not optimized for each plate, because that would prevent a fair comparison between different plates. Essentially, optimizing the gain means that a different charge is imparted to components of the plate reader apparatus so as to provide the maximum spread of the data for that plate. Optimizing the gain for each plate individually would result in setting a different baseline for each plate. Therefore a fixed gain setting of 150 was used across all plates, so that fluorescence readings of different plates could be accurately compared. This setting, determined through previous experiments, allowed for a useful spread of the data on each plate. For the Lipofectamine LTX reagent optimization, the gain setting was 100, as it was part of an earlier experiment.

Chemical Transfection: Optimization II

After the initial optimization, a refined optimization was performed, in which the top ratio/volume conditions indicated by Optimization I were re-tested on more replicates. This was done in order to get a clearer view of which ratio/volume conditions were the most effective. Cryopreserved MSCs were thawed, and cultured in 10% FBS MSC media for 48 hours. They were then plated onto 96-well plates at a low density of 8,000 cells in 100 μ l of 10% FBS antibiotic-free MSC media per well. Use of antibiotic-free media at this stage meant that it was not necessary to change the media immediately prior to transfection, eliminating the risk of removal of some cells by aspiration. Cells were transfected 24 hours after being transferred to the 96-well plates. The transfection solutions were prepared in the same manner as before. Ratio/volume combinations tested in this optimization are shown in Table 2.

Table 2. XtremeGene and FuGene Ratio and Volume Combinations Tested in Optimization II. The volumes for each particular ratio were carefully selected based on the results of Optimization I. Each unique reagent/ratio/volume combination in the table below was tested on multiple different wells.

Reagent	Ratio (μ l reagent to μ g DNA)	Volumes (μ l per well)
XtremeGene HP	1:2	24, 28, 32, 36
	1:1	28, 32, 36, 40
	2:1	20, 24, 28, 32
	3:1	4, 8, 12, 16
FuGene HD	3:2	22.5, 25, 27.5
	4:2	20, 22.5, 25
	5:2	20, 22.5, 25
	6:2	20, 22.5, 25
	7:2	12.5, 15, 17.5
	8:2	17.5, 20, 22.5

Each plate also contained cells-only, DNA-only, and reagent-only controls, in addition to wells that were transfected using a specific set of parameters for the opposite transfection reagent. Each XtremeGene ratio/volume combination was tested on 11 wells, and each FuGene ratio/volume combination was tested on 8 wells. The MSCs were incubated for 48 hours after transfection, before the plates were analyzed.

The preparation for plate reading was also slightly different than in the previous experiment. Instead of aspirating to remove media, which removes some cells, the media was simply tapped out. Then the wells were rinsed with DPBS, instead of HBSS. DPBS does not contain phenol red, so rinsing with DPBS has the additional benefit of not only helping to remove dead cells but also to remove traces of phenol red, which has similar fluorescence properties to RFP. Like the media, the DPBS rinse was removed by tapping, not aspiration. Finally, 100 μ l of DPBS were added to each well and the plates were read with the same settings as before.

Chemical Transfection: Density Optimization

The density of cells at the time of transfection plays an important role in the efficacy of the transfection. Generally, lower densities lead to higher chemical transfection rates, because cell division facilitates the movement of introduced plasmid DNA into the nucleus. To determine optimal density, MSCs were plated at different densities and then transfected with the same reagent/ratio/volume conditions, so that any variation in the transfection efficiency would be a result of density alone.

Cryopreserved MSCs were thawed, cultured for 48 hours, and then plated onto 96-well plates at the following densities: 8, 10, 12, 14, 16, 18, 20, and 22 $\times 10^3$ cells per well. Each

density was used on 24 wells. After growing for 24 hours, the cells were transfected with RFP plasmids using an XtremeGene 1:2 ratio with 32 μ l of transfection solution added per well, following the transfection protocol described previously. After 48 hours, the media was tapped out, wells rinsed with DPBS, and then filled with 100 μ l of a DAPI/DPBS solution (2 μ g DAPI/ml DPBS). DAPI was used to quantify the relative numbers of cells in each well, so that RFP fluorescence relative to total number of cells could be used as a comparison between different densities. Each well was read at 16 different locations in a 4x4 configuration.

Chemical Transfection: Determining Transfection Percent

In order to quantify the actual percentage of cells that were transfected under certain conditions, MSCs were transfected, photographed, and the number of transfected and untransfected cells counted manually. Two XtremeGene ratios were used, and each ratio was used with two different plating densities, to obtain a range of transfection efficiencies.

MSCs at passage 3 were plated onto 6-well plates at two low densities: 237,000 cells/well and 356,000 cells/well, in 3 ml of MSC media. After 24 hours they were rinsed with HBSS, and 3 ml of 10% FBS antibiotic-free MSC media added to each well. Cells were then transfected with RFP plasmids using two XtremeGene ratios, 1:2 and 3:1, according to the transfection protocol described previously. Forty-eight hours later they were rinsed with DPBS, analyzed using the plate reader, and incubated in 4% PFA for 30 minutes. After fixation, the PFA solution was removed and DPBS added to each well. Plates were stored at 4°C and then photographed using phase-contrast and fluorescence microscopy. Fluorescent and total cell numbers were obtained by counting from the photographs.

Chemical Transfection: Effect of Ultrasonication

Ultrasonication has been used as a transfection method, and is sometimes employed in conjunction with electroporation^{17, 18}. It is thought that the ultrasonic waves disrupt the cell membrane and allow DNA to enter the cell. There are no reports of ultrasonication being used with chemical transfection reagents, but it was hypothesized that perhaps exposure to ultrasound could increase the ability of chemical transfection complexes to enter the cell, thereby increasing the efficiency chemical transfection. Hence, chemically-transfected populations of MSCs were exposed to ultrasound for varying periods of time, to determine if this would alter the chemical transfection results.

MSCs at passage 3 were plated onto 12-well plates at a density of 180,000 cells in 1.5 ml of 10% FBS MSC media per well. After 24 hours they were transfected with RFP plasmids using an XtremeGene 1:2 ratio, prepared according to the protocol described previously. Three wells per plate were treated with the following four conditions: DNA, reagent, transfection solution, or no treatment. Each transfected well received 380 μ l of transfection solution. Subsequently, an Ultrasonic Instrument Cleaner (Branson Model 1210, Danbury, CT) was filled with 37°C water and degassed for 5 minutes. Sets of two plates were placed inside double Ziploc bags and floated on the surface of the water. One set of two plates was used for each of the following treatment lengths: 0, 2, 4, 10, or 25 minutes of 47 kHz ultrasound. After 48 hours the plate reader was used to quantify fluorescence in each well.

Physical Transfection: Electroporation

Buffer, voltage, wave shape, cuvette size, number of pulses, time between pulses, cell density, and DNA density are a few of the parameters that can be altered to optimize electroporation. Promising settings for the parameters were obtained through a literature search, particularly from Helledie's and Markar's electroporation recommendations^{25, 39}. These promising settings were tested in Electroporation Trial 1.

Based on an analysis of the results of Trial 1, another set of parameters were tested in Trial 2, to more thoroughly examine the range of parameters that appeared most effective. Likewise, based on the results of Trial 2, an even narrower range of settings was tested in Trial 3, to determine the precise voltage that would result in the highest fluorescence. Additionally, in Trial 3, it was hypothesized that saving the foam that forms in the cuvette after electroporation might result in saving more fluorescent cells. To determine if that was true, the foam in Trial 3 was saved and placed into specific wells that also received the standard amount of electroporated cell solution.

To directly compare the efficacy of electroporation with the efficacy of chemical transfection, plates in Trial 3 were arranged so that half the wells contained electroporated cells and the other half contained cells that were chemically transfected, using one of the top three chemical transfection conditions (as determined through the chemical transfection optimization experiments.) Thus, a plate reader analysis of each plate allowed the fluorescence of electroporated cells to be compared directly with the fluorescence of optimally chemically-transfected cells. Finally, some of the populations of cells from Trial 3 were photographed and counted manually, to determine the transfection percentages for the optimal electroporation condition.

In all electroporation trials, MSCs were removed from cryopreservation, cultured, detached with trypsin, pelleted through centrifugation for 5 minutes at 300 x g, and resuspended in Opti-Mem for counting. Subsequently the MSCs were pelleted a second time, and resuspended in Opti-Mem at a density of 5 million cells/ml. RFP plasmid was added to the cell solution such that the final DNA concentration was 50 µg/ml. Each 2 mm electroporation cuvette was filled to its maximum volume of 400 µl of the plasmid/cell solution (VWR Electroporation Cuvettes Sterile 2mm North American Cat No 89047-208, West Chester, PA). The MSCs were then electroporated with a square wave, using a BTX Harvard Apparatus ECM 830 Electro Square Porator (Holliston, Massachusetts). The electroporation conditions used in Trials 1, 2, and 3 are given in Table 3.

Table 3. Electroporation Conditions Tested in Trials 1, 2, and 3. The Replicates column indicates the number of separate cuvettes that were treated with the corresponding conditions. Conditions tested in Trial 2 are based on results of Trial 1; likewise, conditions tested in Trial 3 are based on results of Trial 2. Asterisks following a Group letter indicate that those conditions were also used on cuvettes containing only cells (as controls). The number of asterisks corresponds to the number of control cuvettes that were treated with those conditions. Cell solution from one cuvette was put into three wells of a six-well plate, or six wells of a twelve-well plate.

Trial Number	Group	Replicates	Electroporation Conditions			
			Volts	Time of pulse	No. of pulses	Interval between pulses (msec)
1	W****	3	130	30 msec	1	n/a
	X****	3	130	30 msec	4	100
	Y	2	505	10 μ sec	5	100
	Z	2	50	1 msec	10	100
2	K*	3	100	30 msec	1	n/a
	L*	3	130	30 msec	1	n/a
	M*	3	160	30 msec	1	n/a
	N*	3	190	30 msec	1	n/a
3	A	3	150	30 msec	1	n/a
	B**	3	160	30 msec	1	n/a
	C	3	170	30 msec	1	n/a
	D	3	180	30 msec	1	n/a
	E	3	190	30 msec	1	n/a
	F	3	200	30 msec	1	n/a

Cells were given a resting period of at least 10 minutes after electroporation before they were plated for culturing. In Trial 1, MSCs were plated in six-well plates at a density of 670,000 cells in 3 ml of 20% FBS MSC media per well. Control wells contained untreated cells, cells cultured in the presence of the plasmid, and cells that were electroporated without any plasmid. Plates were incubated for 24 hours before being observed using phase-contrast and fluorescence microscopy. Then the media was replaced with DPBS and the fluorescence quantified by the plate reader. Each well was read 81 times, in a 9x9 square. Following observation and analysis, the MSCs were incubated for another 24 hours in 10% FBS MSC media, before a second plate reader analysis was obtained.

In Trials 2 and 3, the MSCs were plated onto 12-well plates, at a density of 334,000 cells in 1.5 ml of 10% FBS MSC media per well. Electroporation causes formation of white foam in the cuvette. In Trial 3, this foam was specifically placed in a designated well along with that well's allotment of electroporated cell solution, to determine if saving the foam resulted in higher fluorescence for that well. Also, one well on each plate received approximately 30 μ l of extra electroporated cell solution, to determine if receiving extra cell solution would result in significantly higher fluorescence. After 24 hours, the media was tapped out, wells rinsed with DPBS, and then 1 ml of DPBS added to each well for plate reading. Each well was read 36 times in a 6x6 configuration in Trial 2, and 100 times in a 10x10 configuration in Trial 3.

In Trial 3, twelve wells corresponding to two cuvettes that had been electroporated at 170 volts were additionally photographed at two different locations in the well, with both phase-contrast and fluorescence microscopy. Subsequently, cells were counted from the photographs to obtain an estimate of the percentage of transfected cells. A low count and high count were obtained for the number of fluorescent cells. The low count was more subjective, in that it only

included the “brightest” cells; the high count simply included all visible fluorescent cells, regardless of brightness level. Additionally, an electroporation Trial 4 was performed, in which the cells were electroporated with the same conditions used in Trial 2, except that the electroporation buffer was 20% FBS MSC media instead of Opti-Mem. Trial 4 was carried out to determine whether culture medium could be a satisfactory electroporation buffer.

As part of Trial 3, electroporation was directly compared with the best XtremeGene transfection conditions. Cryopreserved MSCs to be chemically transfected were thawed and cultured at a density of 34,000 cells/cm² in 20% FBS MSC media. After 48 hours they were passaged into 12-well plates at a density of 25,000 cells/cm², and cultured in 10% FBS antibiotic-free MSC media. Cells were only placed in six of the wells of each 12-well plate, and the other six wells left empty. After 24 hours, the media was removed and each well received 1.5 ml of new 10% FBS antibiotic-free MSC media immediately prior to transfection.

XtremeGene transfection solutions of 3:1, 2:1, and 1:1 ratios were prepared according to the manufacturer's instructions. Each particular XtremeGene ratio was used on the six wells containing cells, with the other six wells left empty. MSCs transfected with the 3:1 ratio received 143 µl of transfection solution per well, those transfected with the 2:1 ratio received 238 µl of transfection solution, and those transfected with the 1:1 ratio received 380 µl of transfection solution, corresponding to the optimal volumes for those ratios as determined previously, but scaled up for larger wells. One plate was left untransfected. Following transfection, the cells were cultured for 24 hours.

After 24 hours, electroporated cells were added to the six empty wells of each 12-well plate. These electroporated cells had been thawed 48 hours previously and cultured in 20% FBS MSC media at a density of 34,000 cells/cm². They were electroporated using the protocol

described previously, with parameters set to one 30-msec, 160-volt pulse per 2-mm cuvette. Each cuvette was used to seed five of six empty wells with transfected cells. In the remaining empty well of each plate were placed control non-electroporated cells at the same density as the wells receiving electroporated cells, that is, 335,000 cells per well. Plates were placed in the incubator for 24 more hours before analysis. Thus, the electroporated cells had 24 hours after transfection before they were analyzed, and the chemically-transfected cells had 48 hours after transfection before they were analyzed.

For analysis, media was removed and wells rinsed with DPBS. One ml of DPBS was added to each well and the plate reader used to quantify fluorescence levels. One hundred reads were taken on each well, in a 10x10 square. After plate reading, each well was photographed in two locations using phase-contrast and fluorescence microscopy.

Statistical Analysis

All statistical analysis was performed using R, A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria), with the exception of regression, which was done using Microsoft Excel. Data were compared and analyzed using ANOVA (with Tukey Highly Significant Differences post-hoc testing), the Wilcoxon Rank Sum test, the Wilcoxon Signed Rank test, or Welch's two sample t-test, depending on sample size and distribution of the data.

Normal quantile-quantile plots were used to determine if data was normally-distributed. If the data were not normally-distributed, they were analyzed using a non-parametric test. The powerful Wilcoxon non-parametric tests can be applied to large, normal datasets, but are

especially useful for smaller sample sizes or non-normal datasets. Note that the p-values reported are often less than $2.2\text{e-}16$, since R does not report p-values any smaller than this.

In the density optimization, comparing the raw RFP fluorescence readings of the different densities would not indicate which density had the highest transfection percentage, simply because higher densities have more possible cells that can be fluorescent, so a lower efficiency in a high density could produce a higher RFP reading than a higher efficiency in a lower density. Therefore, the raw RFP values were scaled according to the DAPI reading for that well, and the RFP/DAPI ratio of different densities were statistically analyzed.

Results

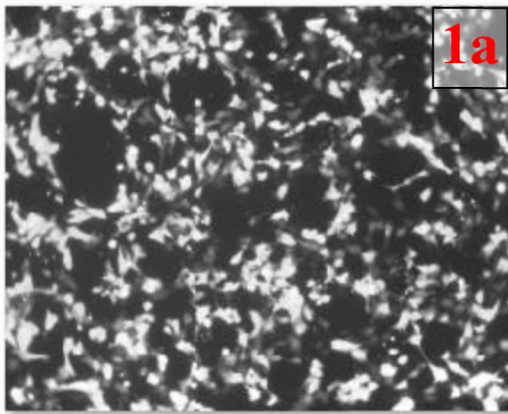
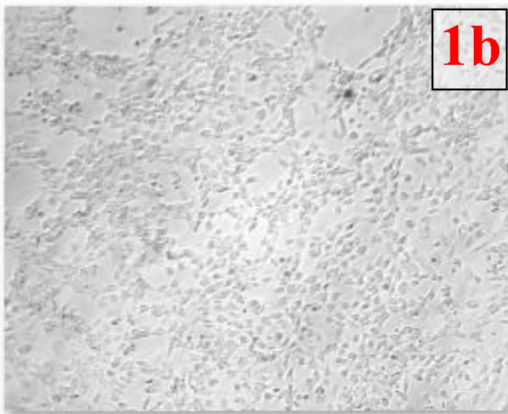
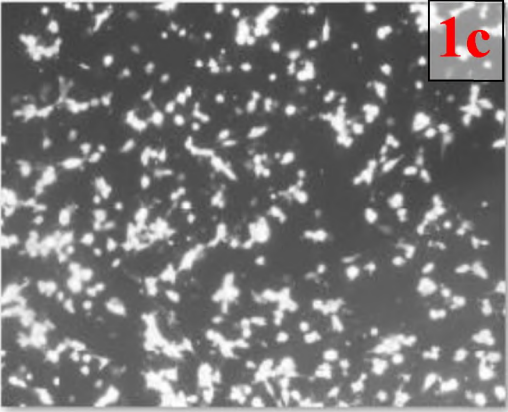
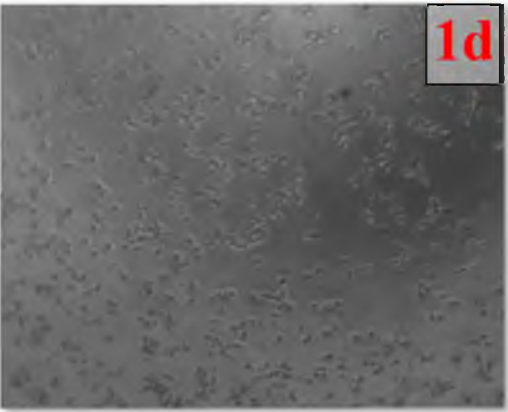
FACS: RFP vs. GFP Expression and Post-FACS Morphology

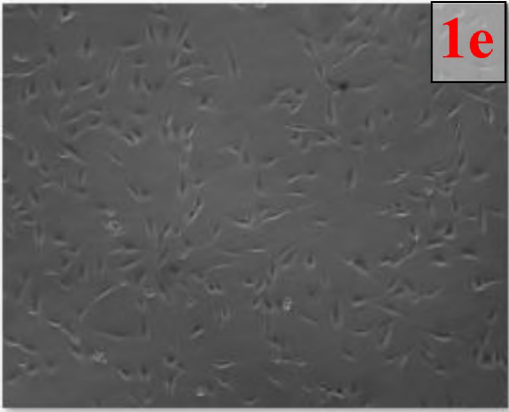
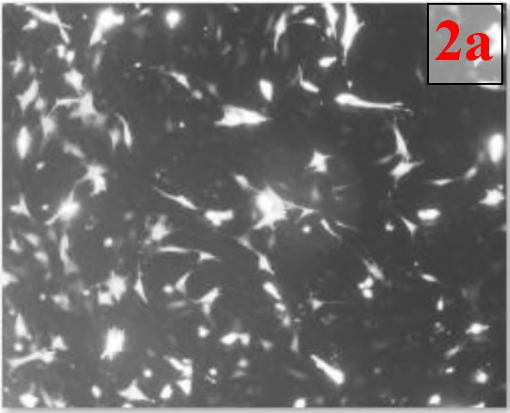
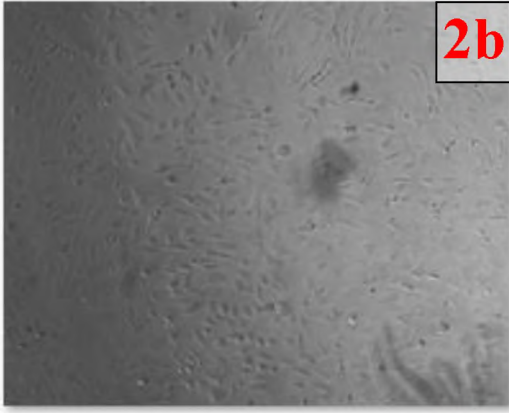

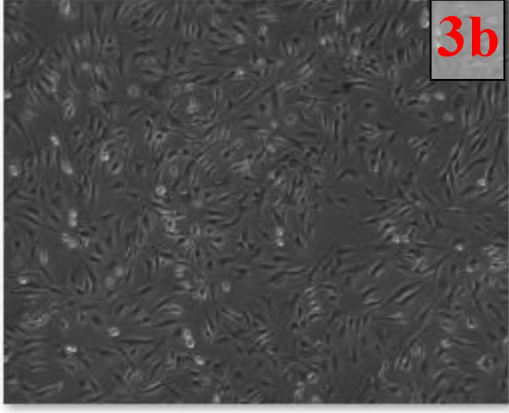
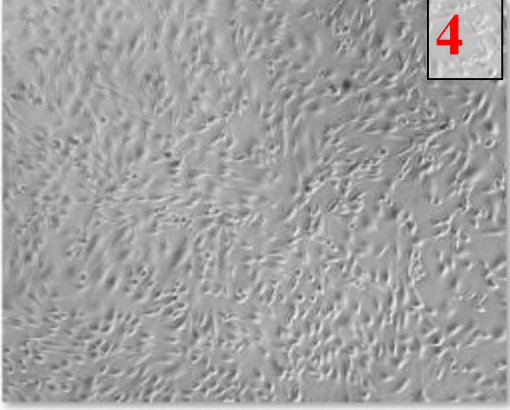
This FACS sorting was carried out to determine which fluorescent protein would be a better reporter gene—more specifically, whether GFP or RFP would be more readily expressed and thus a better indicator of transfection efficiency. RFP-encoding plasmid and GFP-encoding plasmid were co-transfected to determine whether a cell population presented with both plasmids would express one of the plasmids more readily than the other. Overall, more MSCs expressed RFP than GFP. Out of the total cell population that was transfected simultaneously with RFP plasmid and GFP plasmid, 18.3% expressed RFP only, 2.1% expressed GFP only, and 3.3% expressed both GFP and RFP. The overall percentage of transfected cells was 23.7%, determined by summing the aforementioned percentages. This percentage is for an Xtreme 1:1 ratio.

An additional purpose of the FACS test was to determine whether a substantial, healthy population of purified, transfected MSCs could be obtained through fluorescence-activated cell sorting. Only small cell populations of fluorescent cells were obtained, and the morphology of the sorted cells that were actually expressing a transgene was particularly unusual, as shown in Figure 1 (compare Photos 1a – 1d, of expressing, sorted cells, with the remaining Photos.) In general, the cells with the most unusual morphology were the sorted, expressing cells (Photos 1a – 1d), followed by the cells that were transfected but unsorted (Photos 2a, 2b, 3a, and 3b), and finally, the most normal cells were the sorted, un-expressing cells (Photo 1e), and naturally, the unsorted, untransfected control (Photo 4).

Figure 1. Phase-Contrast and Fluorescence Photographs of Expressing/Non-expressing, and Sorted/Unsorted Cells.

“Double” indicates that the cells came from the population that was transfected with both RFP and GFP plasmid simultaneously, which was done in order to compare GFP and RFP expression within the same cell population. “Single” indicates that the cells came from a population transfected with only RFP or only GFP. The “single” cells, which were transfected but not sorted, are a transfection morphology control—these unsorted, expressing cells can be compared with the sorted, expressing cells to determine if there are morphological differences due to sorting. Unsorted, non-expressing cells can also be compared with sorted, non-expressing cells to help determine if there are morphological differences due to sorting. The singly-transfected cell populations were also useful for determining whether RFP or GFP was expressed more effectively. Some cells were neither transfected nor sorted, as a general control. The leftmost column of the table indicates whether the cells were sorted, and what gene(s) they are expressing. Because there were almost no “Double: Sorted: GFP-only” cells after 48 hours, photographs for those cells are not included. Cells were photographed 48 hours after their sorting. Cells that had not been sorted were also photographed at this same time.

Condition	Fluorescence	Phase Contrast
Double: Sorted: Expressing RFP only		
Double: Sorted: Expressing RFP and GFP		

Double: Sorted: Not expressing either transgene (no RFP, no GFP)	Fluorescence photo N/A	
Single: Un-Sorted: RFP-only		
Single: Un-Sorted: GFP-only		
Control: Unsorted, Not transfected		

FACS: Plate Reading Relationship to Percentage of Fluorescent Cells

This second FACS sorting was conducted on cell populations that had been transfected with varying degrees of efficiency, and was used to determine if there was a relationship between the plate reader fluorescence reading and the actual percentage of fluorescent cells in the population. Cells were analyzed with a plate reader and subsequently sorted on a FACS Aria. The percentage of fluorescent cells in the population was provided by the cell sorter after sorting.

There was a strong linear relationship between the average plate reader fluorescence reading for a plate, and the percentage of fluorescent cells for that plate, which is shown in Figure 2 ($R^2 = 0.98$). Each point on the graph corresponds to the next-highest transfection solution volume used; the different transfection solution volumes did lead to significant differences in fluorescence levels, depicted in Figure 3 (ANOVA, followed by Tukey-Kramer HSD Test, $p < 0.0001$) for every pairwise comparison of volumes except for the last two, 28 μl vs. 32 μl .

Figure 2. Percentage of Fluorescent Cells vs. Plate Reader Fluorescence Reading.

Fluorescence readings from the plate reader are strongly correlated with percentage of fluorescent cells as determined by FACS. Higher plate reader readings correspond to higher percentages of fluorescent cells. The percentage of transfected cells is not particularly high here because this experiment was carried out before any of the transfection protocols had been optimized. Also, the plate reader gain setting used here was slightly different from the gain setting used in some other experiments—which, though it changes nothing about the plate reader's efficacy as an instrument and only alters the absolute numerical value of the output, means that the numerical fluorescence readings in other experiments should not be translated into a transfection percentage using this graph.

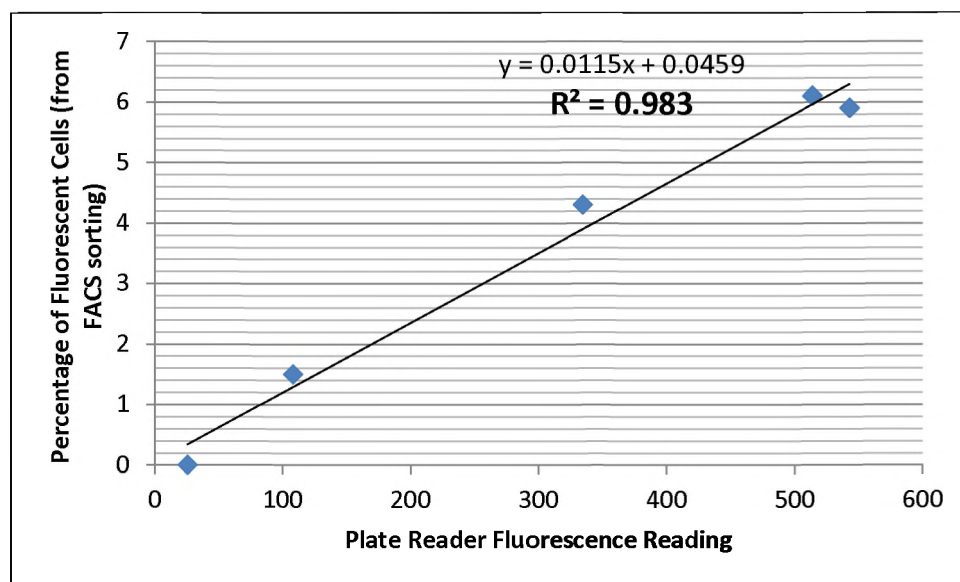
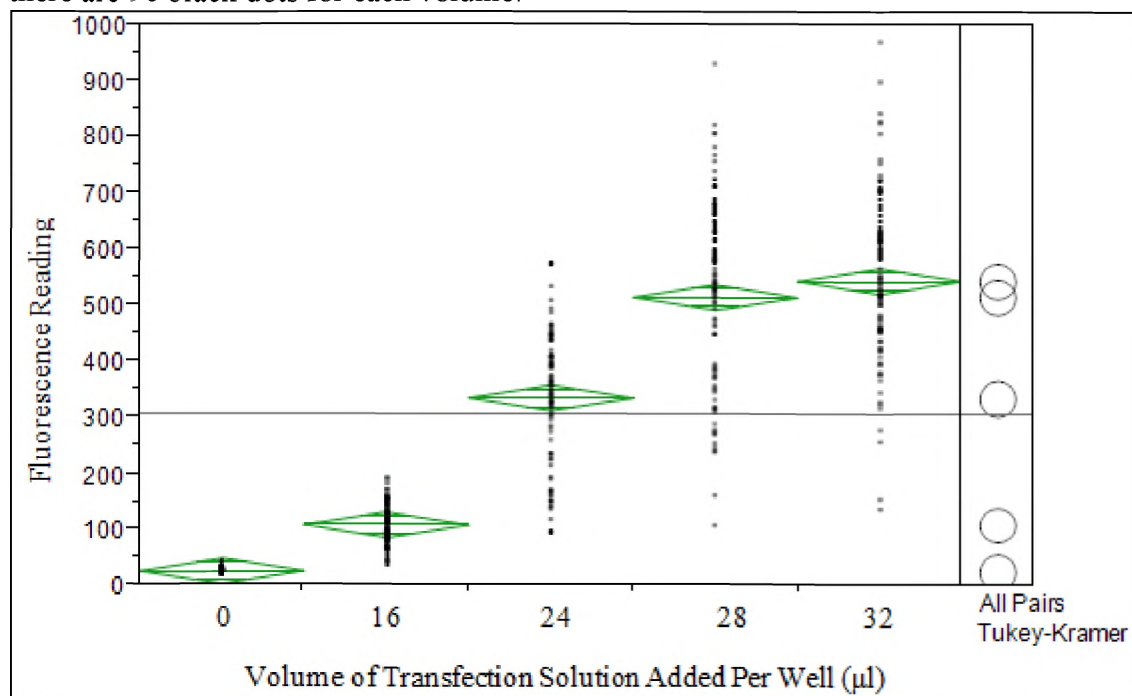


Figure 3. Distribution of Fluorescence Readings for Wells of 96-Well Plates Transfected by Different Volumes of XtremeGene 1:2 Transfection Solution. Each individual well reading is represented by a single black dot. Each transfection solution volume was used on 96 wells, so there are 96 black dots for each volume.



Chemical Transfection Optimizations I & II: Optimization of Reagent, Ratio, and Volume

Chemical Transfection: Optimization I

The initial optimization across all reagents, ratios, and volumes, indicated that some sets of conditions resulted in higher fluorescence than others. A comparison of all fluorescence readings of each reagent/ratio combination indicates that three pairs of FuGene ratios significantly differed from each other (Wilcoxon Rank Sum, 3:2 vs. 8:2, and 4:2 vs. 8:2, $p < 0.05$) while six pairs of XtremeGene ratios significantly differed from each other (Wilcoxon Rank Sum, 1:4 vs. 2:1, 3:1, 4:1, and 1:1 vs. 2:1, 3:1, 4:1; $p < 0.03$). However, when FuGene ratios were compared to XtremeGene ratios, there were 22 significantly different pairings out of 36 total pairings (Wilcoxon Rank Sum, $p < 7.53\text{e-}05$ for 18/22 pairwise comparisons, and $p < 0.03$ for all 22 of the pairwise comparisons). XtremeGene overall has higher fluorescence readings than FuGene overall (Wilcoxon Rank Sum, $p = 9.89\text{e-}7$). Additionally, XtremeGene and FuGene each had significantly higher fluorescence readings than the controls (Wilcoxon Rank Sum, $p < 2.2\text{e-}16$).

The FuGene ratios all tended to have peak fluorescence around a transfection solution plating volume of 22.5 μl per well, as indicated in Figure 4. However, the XtremeGene ratios each had different volumes at which fluorescence was maximal, appearing to follow a third-order polynomial ($R^2 = 0.95$) and with higher ratios showing lower most effective volumes, as shown in Figures 4 and 5. Lipofectamine LTX had peak fluorescence at a 4:1 ratio, 10 μl plating volume (Appendix 1, Table S1).

Figure 4. Chemical Transfection Optimization I: Trends in Fluorescence Readings Across All Ratios and Volumes for FuGene and XtremeGene Reagents.

The number provided for each ratio is the microliters of reagent that correspond to one microgram of DNA, which is why the XtremeGene 1:4 ratio is listed as 0.25 and the 1:2 ratio as 0.5. Notice the difference in scale on the y-axis of the two graphs; XtremeGene clearly results in higher fluorescence levels than FuGene.

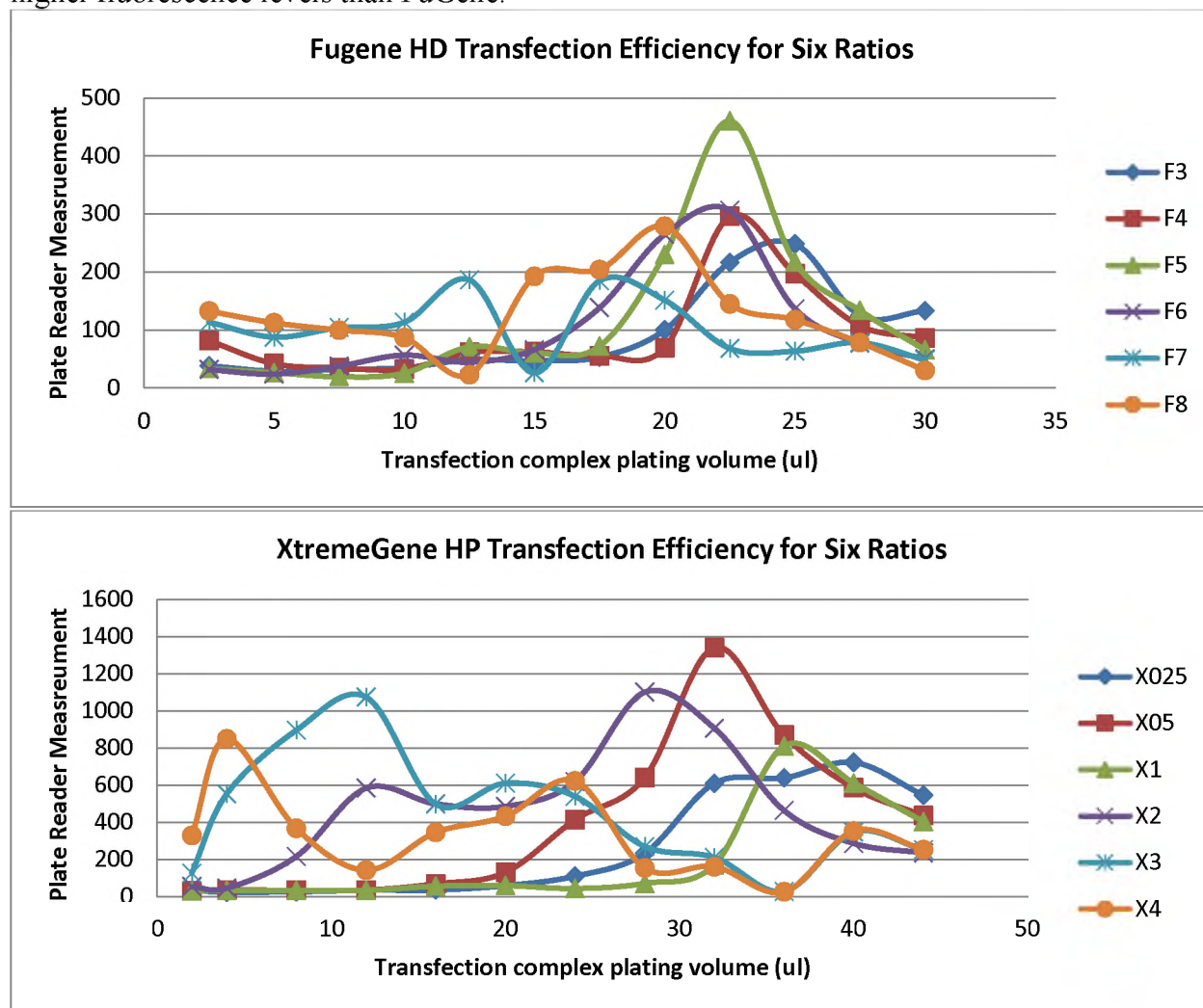
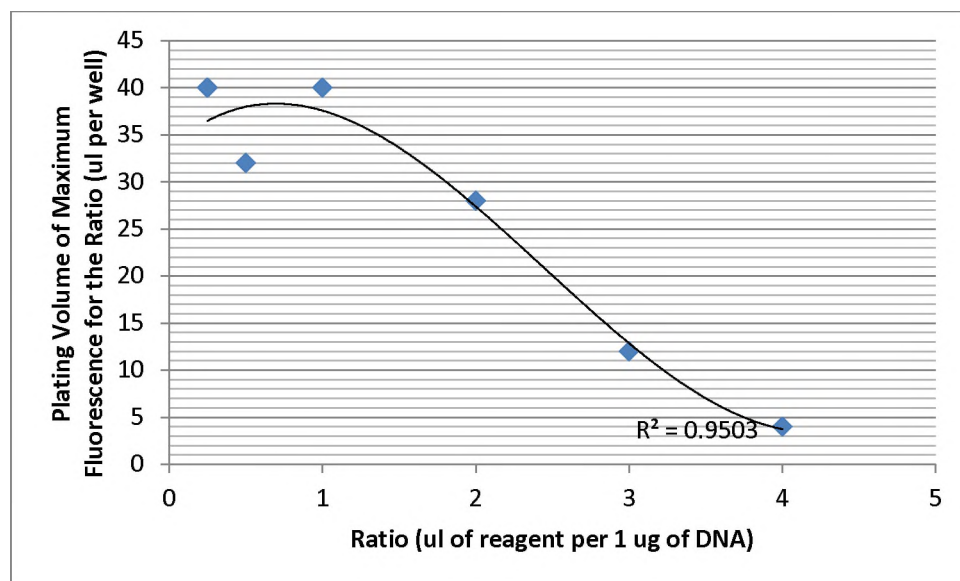


Figure 5. Volume At Which Maximum Fluorescence Occurred Across Six XtremeGene Ratios. For each ratio, the volume at which the highest fluorescence reading was achieved is plotted against the ratio. Lower ratios have generally higher optimal plating volumes.



Chemical Transfection: Optimization II

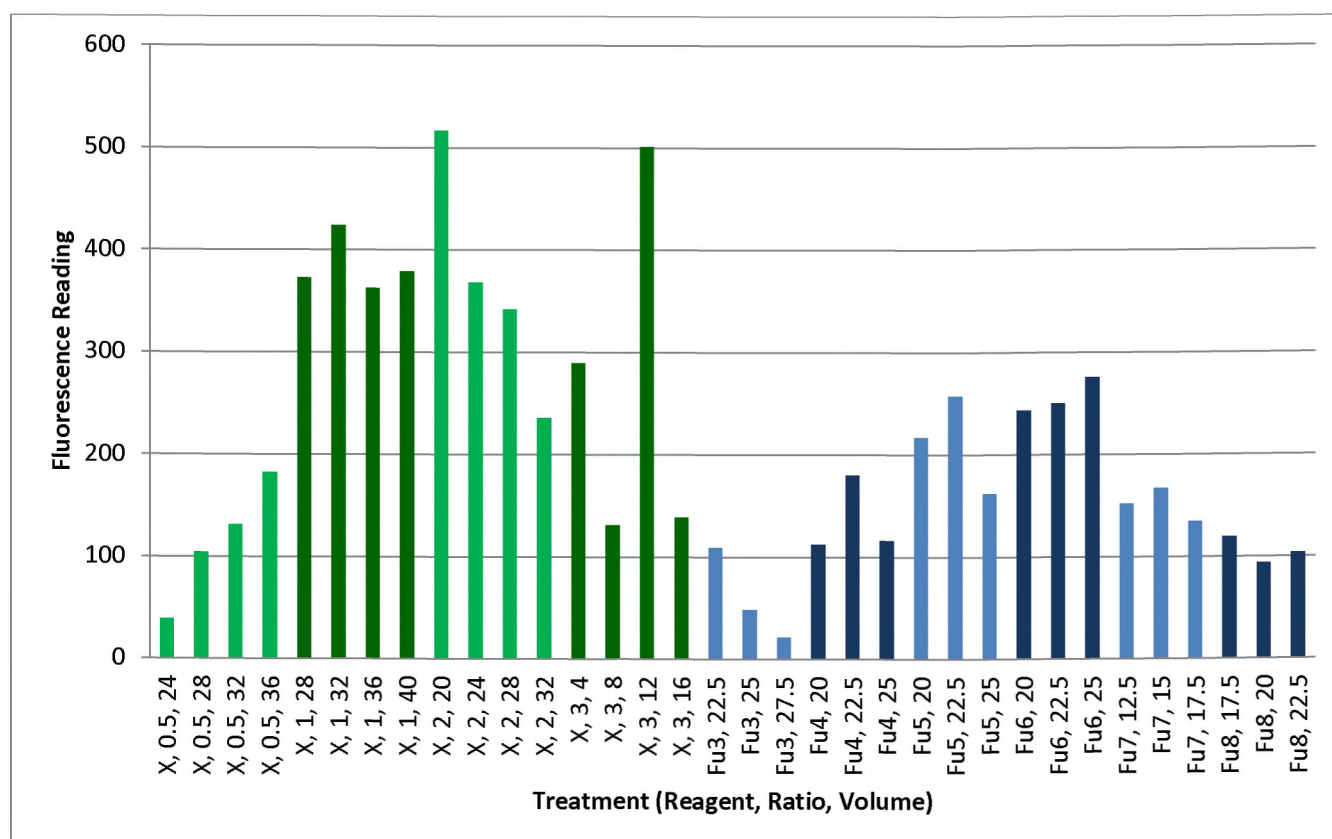
This experiment focused on the transfection conditions that resulted in the highest fluorescence readings in Optimization I, and evaluated these conditions with more replicates. XtremeGene ratios of 1:2, 1:1, 2:1, and 3:1 were tested for the top four volumes in each ratio; FuGene ratios of 3:2 to 8:2 were tested for the top three volumes in each ratio.

FuGene and XtremeGene again both had significantly greater fluorescence than controls, and, as apparent in Figure 6, XtremeGene had significantly greater fluorescence than FuGene (Wilcoxon Rank Sum, $p < 2.2e-16$). Within XtremeGene, each ratio was significantly different from each other ratio (Wilcoxon Rank Sum, $p < 0.0001$), with the exception of the 2:1 ratio against the 1:1 ratio. Pooling the 2:1 and 1:1 XtremeGene data and comparing it with the other XtremeGene data showed that the fluorescence readings of 2:1 and 1:1 ratios were higher than the readings of the 3:1 and 1:2 ratios (Wilcoxon Rank Sum test, $p < 2.2e-16$). For the 2:1 and 1:1 ratios, the only volumes that had significantly different fluorescence readings from any others were the 2:1 ratio 20 μ l volume, the 2:1 ratio 32 μ l volume, and the 1:1 ratio 32 μ l volume (Wilcoxon Rank Sum, $p < 0.029$).

Figure 6. Chemical Transfection II, FuGene and XtremeGene Average Fluorescence Readings for Top Ratio/Volume Combinations.

The second chemical transfection optimization tested the top four volumes of each of the top four ratios for XtremeGene, and the top three volumes of each of the six ratios for FuGene. The average fluorescence reading for each ratio/volume combination is depicted below.

XtremeGene treatments are shown in green, and FuGene treatments are shown in blue. The alternating light and dark colors within each ratio emphasize the grouping by ratio. Ratios increase from left to right within a reagent, and volume increases from left to right within a ratio. XtremeGene ratios are written as the μl of reagent that would correspond to 1 μg of DNA (hence, 0.5 refers to the 0.5:1 ratio, which is elsewhere referred to as the 1:2, ratio), while FuGene ratios are written as the μl of reagent that would correspond to 2 μg of DNA. Volumes are provided as μl added per well of a 96-well plate, where each well initially contains 100 μl of media.



Chemical Transfection: Density Optimization

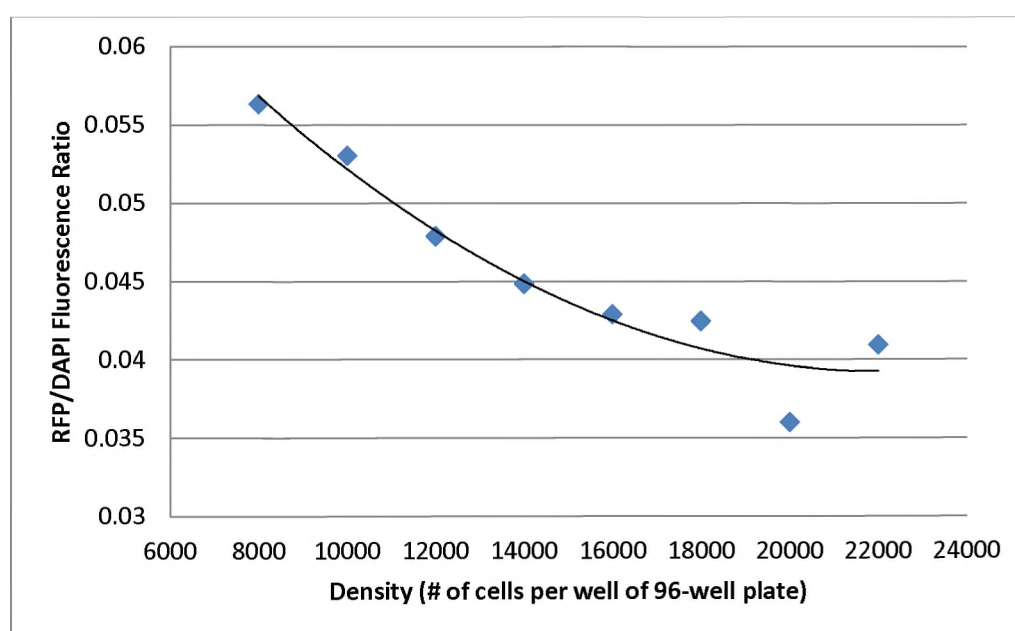
This experiment was performed to determine the effect of cell density on chemical transfection efficiency. MSCs were plated across a wide range of densities, and these separate populations transfected with the same set of conditions, to see how the density would impact the percentage of fluorescent cells obtained.

In this experiment it was important to compare the relative RFP/DAPI ratios rather than the raw RFP readings, because the deliberate variation in total cell numbers was guaranteed to skew the final sizes of the populations (see Statistical Analysis in Methods, and Figure 7 below for more information.)

As a check, it was determined that the wells that received more cells had higher DAPI fluorescence readings (linear regression, $R^2 = 0.94$). The lowest density (8×10^3 cells/well) had a significantly higher RFP/DAPI fluorescence ratio than the second-highest density (20×10^3 cells/well) (Welch Two-Sample t-test, $p = 0.0009$). Additionally, the lowest density (8×10^3 cells/well) had significantly higher RFP/DAPI ratio than the highest density (22×10^3 cells/well) (Welch Two-Sample t-test, $p = 0.005$). Overall, the lower densities had greater RFP/DAPI ratios than the higher densities, a trend that can be easily seen in Figure 7.

Figure 7. Average RFP/DAPI Fluorescence Ratio for Transfection at Different Cell Plating Densities.

Because the RFP fluorescence reading is indicative of the number of transfected cells in a well, and the DAPI fluorescence reading is indicative of the total number of cells, the RFP/DAPI ratio is related to the percentage of fluorescent cells. Note that although comparing RFP/DAPI ratios is useful for understanding relative transfection percentages, the actual value of the ratio itself does not equal the exact transfection percentage, due to the differing properties of the fluorescent molecules. The reason the RFP/DAPI ratio was used to analyze transfection success, rather than the RFP reading alone, is that the final cell populations have different sizes according to the differing densities. Therefore, if RFP reading alone were used, higher densities would have an “unfair advantage” by virtue of simply having more cells present. Here it can be seen that higher densities have a smaller RFP/DAPI ratio, indicating a smaller percentage of fluorescent cells than the lower densities.

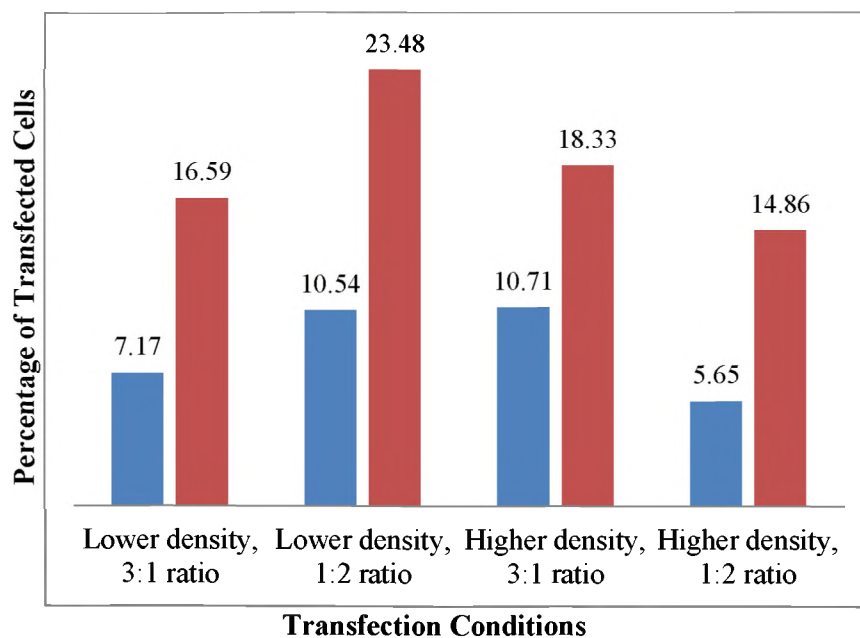


Chemical Transfection: Determining Transfection Percent

Overall 16,400 cells were counted, to determine the transfection percentages for two densities and two transfection ratios, shown in Figure 8. The highest transfection percentage was 24%, for the lower density XtremeGene 1:2 ratio.

Figure 8. Transfection Percentage for Two XtremeGene Ratios and Densities.

The blue bar indicates a lower estimation of the transfection percentage, obtained by a count of only the brightest fluorescent cells. The red bar indicates the transfection percentage as determined when every fluorescent cell was counted, regardless of its relative brightness.



Chemical Transfection: Effect of Ultrasonication

Chemically-transfected cells were ultrasonicated for differing periods of time, to determine how ultrasound would affect the transfection efficiency. The fluorescence readings for cell-only, DNA-only, and reagent-only controls were negligible, even if the plate was ultrasonicated. For the chemical transfection solution treatments, wells that were ultrasonicated for 4 minutes had significantly higher fluorescence than non-ultrasonicated transfection wells (Wilcoxon Rank Sum, $p=0.021$). Transfection wells treated for 25 minutes had significantly lower fluorescence than non-ultrasonicated transfection wells (Wilcoxon Rank Sum, $p=0.032$).

Physical Transfection: Electroporation

Trial 1:

This was the initial optimization, which tested a variety of conditions to determine what parameters merited further investigation. The X group (130 V, four 30-msec pulses), including both X samples and X controls, were completely dead after 24 hours. Out of the groups where cells did survive, the only group with fluorescent cells was group W (130 V, one 30-msec pulse). This group had fluorescence readings that were significantly different from the fluorescence readings of the non-electroporated controls (Wilcoxon Rank Sum, $p=0.0002$). Groups Y (505 V, five 10- μ sec pulses) and Z (50 V, ten 1-msec pulses) did not have any fluorescent cells present.

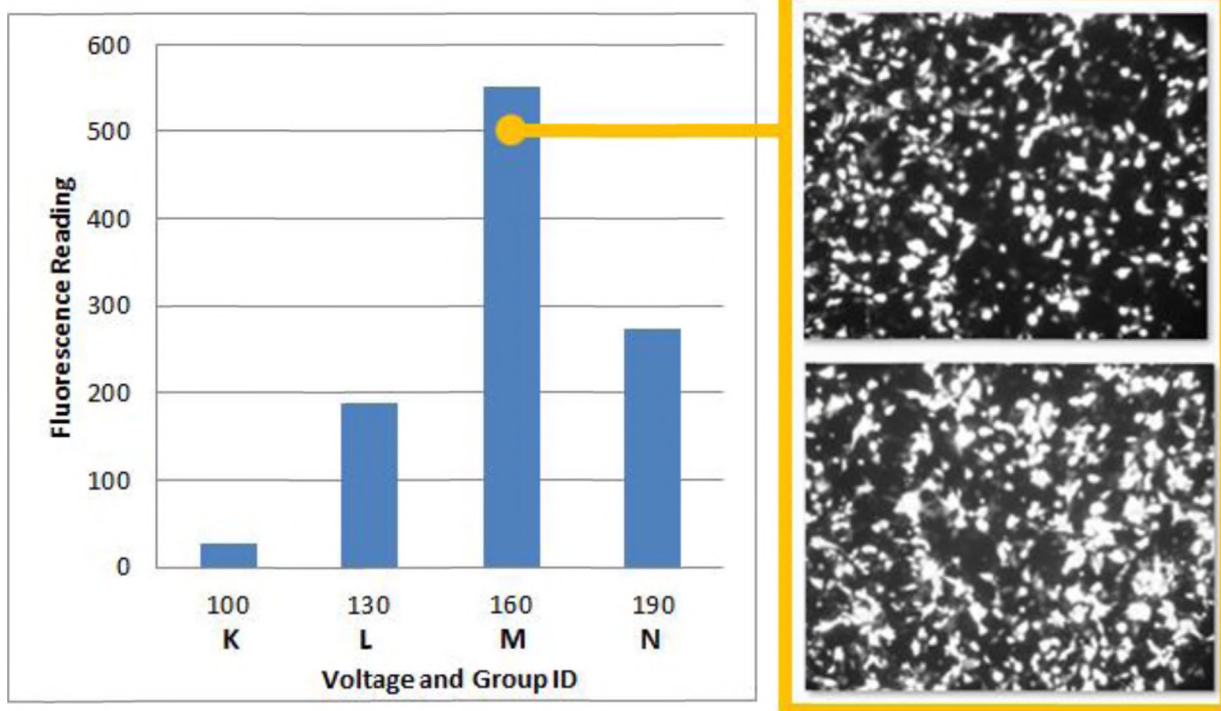
Trial 2:

Here, parameters in a range suggested by Trial 1 were tested in more detail. Groups K (100 V, one 30-msec pulse), L (130 V, one 30-msec pulse), M (160 V, one 30-msec pulse), and N (190 V, one 30-msec pulse) all had fluorescence that was significantly greater than the controls (Wilcoxon Rank Sum, $p < 2.56e-10$). All groups were significantly different from each

other (Wilcoxon Rank Sum on each pairwise comparison, $p < 0.037$). Group M (160 V) had significantly higher fluorescence than all other groups, as suggested by Figure 9 (Wilcoxon Rank Sum test, $p=0.0012$ for 190V, $p=6.45e-09$ for 100V, $p=4.32e-07$ for 130V).

Figure 9. Fluorescence Readings of Cell Populations 24 Hours After Electroporation At Four Different Voltages, and Photographs of Highest Fluorescence Populations.

The peak in the graph occurs at 160 volts. Photographs were taken in Trial 2, twenty-four hours after electroporation. The two photographs shown are from populations electroporated at 160 volts.



Trial 3:

This test was designed to further narrow the voltage range based on results of Trial 2; to determine the effect of saving the electroporation foam; and to directly compare the transfection efficiency of electroporation with the transfection efficiency of the optimal chemical transfection parameters.

Every electroporated group had higher fluorescence than the controls (Wilcoxon Rank Sum, $p < 5.77e-07$). When fluorescence readings from different voltages were compared, the readings for the Group C (170 V) were significantly higher than the readings for all other voltages, as indicated in Figure 10 and 11 (Wilcoxon Rank Sum, $p < 0.0073$). Additionally, the readings for the Group D (180 V) were significantly higher than the readings for Groups B, E, and F (160, 190, and 200 V) (Wilcoxon Rank Sum, $p < 0.013$).

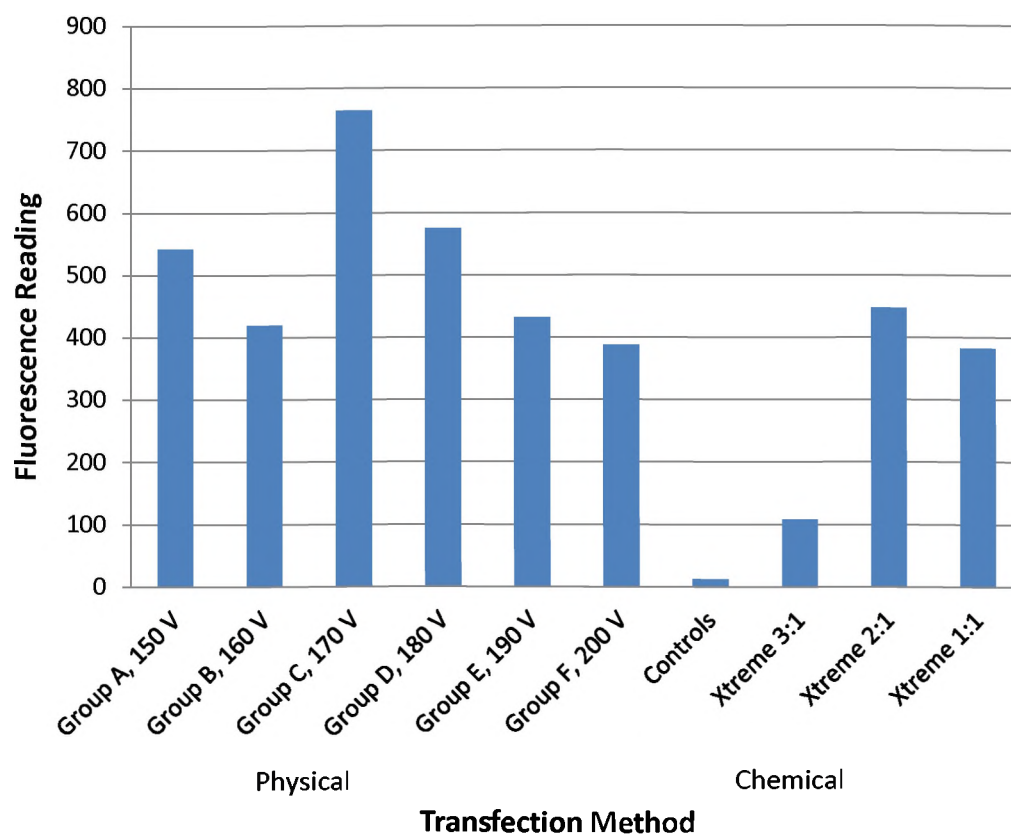
Figure 10. Results of Statistical Analysis Indicating that Group 170 Volts Had Higher Fluorescence Than All Other Voltages.

Wilcoxon Rank Sum p-values are shown, for the alternative hypothesis that Group C (170 volts) has brighter fluorescence than the group noted in the leftmost column.

Group (Voltage)	Wilcoxon p-value
A (150)	0.00726
B (160)	0.000197
D (180)	0.0064
E (190)	1.33e-05
F (200)	1.754e-06

Figure 11. Fluorescence Readings for Six Electroporation Voltages and the Top Three XtremeGene Transfection Ratios.

The highest fluorescence readings were achieved by the group electroporated at 170 volts. The XtremeGene 2:1 and 1:1 ratios have fluorescence readings similar to those of the groups electroporated at 160, 190, and 200 volts.



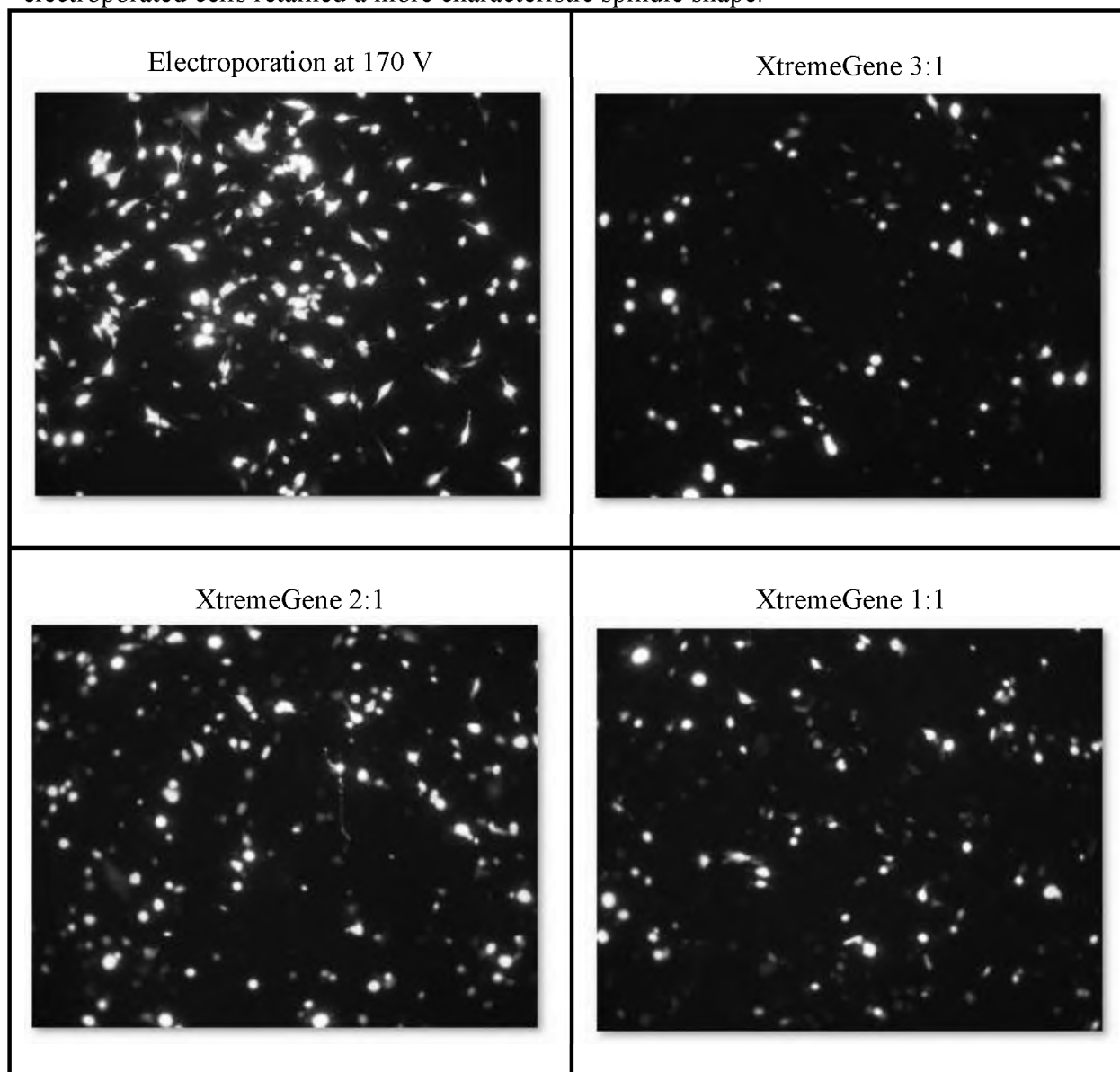
Next, fluorescence readings for wells were paired according to source cuvette, and separated by whether the well had the standard amount of electroporated cell solution added, or also had cell foam or extra solution added. The wells that had received foam in addition to cell solution had higher fluorescence than the wells that had only received the standard amount of cell solution (Wilcoxon Signed Rank Test, $p=0.0017$). The wells that had received extra solution had higher fluorescence than the wells that had only received the standard amount of cell solution (Wilcoxon Signed Rank Test, $p=3.815e-06$). And, the wells that had received extra solution had higher fluorescence than the wells that had received foam (Wilcoxon Signed Rank Test, $p=0.002$).

The electroporation conditions of Trial 3 for 170 volts resulted in 40.1% of cells being “brightly” fluorescent (2356 brightly fluorescent cells out of 5876 total cells), and 53.6% of cells having fluorescence (3148 fluorescent cells out of 5876 total cells). In Trial 4, when the cells were electroporated in 20% FBS MSC media to determine if media was a satisfactory buffer, visual observation indicated that almost no fluorescent cells were present whatsoever.

In the direct comparison of XtremeGene to electroporation transfections, both XtremeGene and electroporation resulted in fluorescence levels significantly higher than that of the controls, which included untreated cells, cells treated with naked DNA, and cells electroporated by one 30-msec pulse of 160 V without DNA present (Wilcoxon Rank Sum, $p < 1.02e-07$). Cells transfected with the Xtreme 2:1 ratio had higher fluorescence than those transfected with the 3:1 ratio and those transfected with the 1:1 ratio (Wilcoxon Rank Sum, $p < 0.05$). The electroporation with 170 volts resulted in significantly greater fluorescence levels than any of the XtremeGene transfection ratios (Wilcoxon Rank Sum, $p < 0.027$). Photographs of cells electroporated at 170 volts are shown in Figure 12, along with chemically-transfected cells.

Figure 12. Fluorescence Photographs of Cells 24 Hours After Electroporation at 170 Volts, and 48 Hours After Chemical Transfection by XtremeGene.

The photographs illustrate the same trend apparent from the plate reader fluorescence quantification—namely, that electroporation at 170 volts resulted in the most fluorescent cells, followed by XtremeGene ratios 2:1, 1:1, and last of all 3:1. Additionally, it can be seen that chemical transfection led to rounder cell morphology, whereas a substantial proportion of the electroporated cells retained a more characteristic spindle shape.



Discussion

Fluorescence-Activated Cell Sorting

For a transfection reporter gene, tdTomato appears to be more effective than eGFP. When cells were transfected with equal amounts of both plasmids, RFP was expressed more highly, and when separate populations were transfected with one plasmid or the other, there were far more fluorescent cells in the RFP-transfected population than the GFP-transfected population. One reason for this disparity could be a difference in protein brightness—after all, tdTomato is advertised as being a particularly vibrant protein. If the RFP was brighter than the GFP, then in the dually-transfected population, the FACS Aria may have categorized some GFP-transfected cells as untransfected, and additionally could have categorized some dually-expressing cells as expressing only RFP. However, visual observation of cells that had been transfected with only RFP or only GFP also showed a dramatic difference in the proportions of cells expressing RFP as compared to GFP, suggesting that for whatever reason MSCs expressed tdTomato more effectively. Hence, the RFP plasmid was chosen as the reporter gene for the optimization experiments.

In addition to suggesting the best fluorescent protein to use, the FACS results also provided more motivation for optimizing transfection. Fluorescence-activated cell sorting of a cell population transfected at medium efficiency does not appear to be the most efficient way of obtaining a healthy, pure population of low-passage-number equine MSCs all expressing a gene of interest. The population ultimately obtained is small, and cells are thoroughly stressed by the sorting process. In spite of coating the collection tubes with BSA and using a sorting buffer recipe that was designed to maximize cell survival, the morphology of the MSCs after sorting was unusual for a number of days after sorting. Also, undoubtedly some of the transfected cells

were lost in the sorting process, because cells with lower fluorescence levels might not reach the set cutoff point. It is more efficient to simply use an effective transfection method to begin with. Based on the FACS measurement of the proportion of fluorescent cells transfected by different volumes of XtremeGene 1:2 ratio, as well as the plate reader output for those same cell populations, it also became clear that transfection solution volume was one of the key parameters to optimize, since different volumes resulted in dramatically different transfection efficiencies.

The FACS sorting results also suggest that expression of a transgene may have a more dramatic effect on cell morphology than FACS sorting itself, since the cells that were expressing a transgene but were not sorted had a more unusual morphology than the cells that were sorted but not expressing a transgene. However, FACS sorting still clearly has an effect, since the cells with the most unusual morphology were the ones that were both sorted and expressing a transgene.

As evidenced by the FACS/plate reader standard curve, the plate reader appears to be an effective way of measuring relative gene expression among different populations of cells. The value given by the plate reader has a strong linear relationship to the actual percentage of fluorescent cells present, with higher fluorescence readings indicating a proportionally higher percentage of expressing cells. Thus, both the use of RFP as a reporter gene and the use of the plate reader to quantify transfection efficiency are strongly supported by the results of the FACS tests.

One detail that is worth discussing is the plate reader's potential inability to distinguish between large numbers of dim cells and high numbers of bright, tiny cells or vesicles. This would be a complication if the different transfection methods resulted in dramatic variation in the brightness or sizes of cells observed. However, it seems qualitatively that there is no substantial

difference in size or brightness variation between chemically-transfected cells and electroporated cells. Possibly, the chemically-transfected cells may be slightly rounder than electroporated cells, and may expel more fluorescent vesicles, probably to clear out reagents. But even with their “unfair advantage”—since, after all, the quantity of interest is proportion of fluorescent cells and not proportion of fluorescent vesicles—the chemically-transfected cells still had significantly lower fluorescence than the electroporated cells. Whether or not there actually is a difference in size or brightness of chemically-transfected cells compared to electroporated cells is not obvious, so there appeared to be no need to conduct a of quantitative assessment of this potential variation. Perhaps, if other transfection methods had been tested that resulted in sharp changes of size or brightness variation, the plate reader strategy would have had to be modified.

A minor point of conflict between the data from two of the experiments is the relative fluorescence level of the 28 μ l volume and the 32 μ l volume for an XtremeGene 1:2 ratio. In Chemical Optimization I, use of 32 μ l per well led to approximately twice the fluorescence level of 28 μ l per well. However, when the plate reader’s accuracy was being verified through FACS, there was no significant difference between the fluorescence resulting from use of 32 μ l and use of 28 μ l. Most likely, this is simply a reflection of the small sample size used in Optimization I ($n=3$) against the much larger sample size used for each volume in the plate reader test ($n=96$), and was part of the motivation for conducting the second, refined chemical optimization. Chemical Optimization II included larger sample sizes than Optimization I. It is noteworthy that the general trend of increasing expression as the transfection solution volume increases from 16 μ l up to 32 μ l was the same between the two tests.

Chemical Transfection Optimizations

XtremeGene proved to be a better transfection reagent than FuGene HD. The most effective ratios for transfecting equine MSCs are the XtremeGene 2:1 and 1:1 ratios. According to the data from both Optimization II and the final direct comparison of XtremeGene 1:1, 2:1, and 3:1 ratios to electroporation, the best overall set of conditions for chemical transfection is an XtremeGene 2:1 ratio, with a volume added that is equivalent to 20 μ l of transfection solution added per well of a 96-well plate, where each well initially contains 100 μ l of media. To ensure satisfactory transfection, the XtremeGene used should be as new as possible, since it was discovered that expired XtremeGene does not work (Appendix 1, Table S2). It is also worth noting that Lipofectamine LTX & PLUS reagent could still prove more effective than XtremeGene; though it had promising initial results, Lipofectamine was not optimized further only because of delays encountered when attempting to obtain more of the reagent.

In addition to choosing a useful reagent, ratio, and volume, it is important to use low density of cells, to achieve maximum chemical transfection efficiency. When compared to other densities, the lowest density of 8,000 cells/well had the highest RFP/DAPI fluorescence ratio, indicating that it had the highest percentage of cells transfected. It is possible that even lower densities than this could produce higher percentages of fluorescent cells, an avenue that could easily be explored in further experiments.

The effects of ultrasound on chemical transfection efficiency are not clear. Although ultrasonication for 4 minutes did appear to increase the transfection efficiency, ultrasonication for 25 minutes had the opposite effect. Perhaps, 4 minutes was long enough to facilitate entry of transfection complexes into cells, but 25 minutes was so long that it caused permanent damage to cellular structures and, through killing cells, resulted in lower transfection efficiency. The

frequency used in this experiment, 47 kHz, is not a frequency that is typically used, nor are ultrasonic instrument cleaners the usual method of sonoporating cells. Checking whether other frequencies at other durations would increase chemical transfection efficiency would certainly be interesting. Additionally, use of microbubbles or other sonoporation reagents may lead to discovery of another highly efficient nonviral transfection method for equine MSCs. Already, the non-optimal XtremeGene 1:2 ratio transfection percentage of 24% is on the higher end of MSC chemical transfection efficiencies previously reported (Table 1).

Physical Transfection: Electroporation

Electroporation is the best transfection method out of all methods studied here. The optimal set of parameters for transfecting equine MSCs are concentrations of 5 million cells/ml and 50 µg plasmid/ml, with 1 square-wave 30-msec 170-volt pulse. It is critical that Opti-Mem be used as the buffer, since use of MSC media as the buffer results in very poor transfection efficiency. Also, saving the “foam” that forms in the cuvette results in a greater number of fluorescent cells. Most likely, the foam is composed of cells. The maximum equine MSC electroporation efficiency achieved, 54%, is on the higher end of maximum MSC electroporation efficiencies previously reported, which include 30-40% and 90% for humans, <2% in pig, and 29% in rat^{12, 25, 31, 40}.

It would be useful to determine if electroporation or chemical transfection of equine MSCs affected their stem cell characteristics in any way. In a study on rats, electroporation did not affect MSC multidifferentiation; they were successfully differentiated down osteoblast, adipocyte, and chondrocyte pathways³¹. However, in a separate study, Lipofectamine-transfected human MSCs characteristics were altered by the chemical treatment²⁵. Information about the

effects of transfection on MSC “stemness” could provide additional help in choosing which method to use.

It would also be worthwhile to study the effects of electroporation and XtremeGene-mediated transfection on gene expression in MSCs, since the transfection methods themselves can cause differential gene expression that is unrelated to the actual gene that is transferred to the cell. One study found greater than a tenfold change in the number of differentially expressed transcripts, depending on which transfection reagent they used, because transfected cells can react to the transgene as they would to a viral infection even when viruses themselves are not used for gene delivery⁴¹. Potentially, the unique ways in which gene expression is altered between these methods could change the therapeutic potential of the MSCs, and provide new insight as to which method could be better in a given situation. However, if transfection efficiency is the primary concern, then electroporation is the best method.

Equine MSCs are a valuable cell type, and provide opportunities to learn more about horse health and, by inference, human health. These optimized transfection methods, especially the set of highly effective electroporation parameters, will undoubtedly be useful to the multitude of researchers who wish to genetically modify equine MSCs.

List of Abbreviations

- AAV: Adeno-associated virus
- BSA: Bovine serum albumin
- CMV: cytomegalovirus
- DAPI: 4',6-diamidino-2-phenylindole
- DPBS: Dulbecco's Phosphate-Buffered Saline
- EDTA: Ethylenediaminetetraacetic acid
- FACS: Fluorescence-activated cell sorting
- FBS: Fetal bovine serum
- GFP: Green fluorescent protein
- HBSS: Hank's Balanced Salt Solution
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HDS: Highly Significant Differences
- MSC: Mesenchymal stem cell
- PFA: paraformaldehyde
- RFP: Red fluorescent protein (here refers to tdTomato)
- RT-PCR: real-time polymerase chain reaction

Conflict of Interest

The author certifies that there are no conflicts of interest regarding the material discussed in this manuscript.

Acknowledgements

First I wish to thank my God, my parents, my grandparents, and Rebecca, Joseph, and Catherine, for their continuous support in my endeavors at Cornell, including all the work that has gone in to this thesis. Without them, this would not have been possible.

I am very grateful to Dr. Alan Nixon, for the excellent opportunity to work in his laboratory, his scientific advice, and his valuable suggestions for improving this manuscript. I thank the other reviewers of my thesis for their input as well.

I would also like to thank Jeremy Ackerman-Yost and Michael Scimeca for teaching me countless laboratory techniques. Mike also patiently answered my endless stream of questions, which was immensely helpful throughout the design, execution, and write-up of this project. I also thank him for his feedback on this manuscript. I am indebted to everyone in the Nixon lab for their suggestions and good conversation, as well as to Lavanya Sayam for her explanations regarding FACS.

Additionally, I thank Dr. John Hermanson, for introducing me to research when I was a freshman, inspiring me to keep at it, and providing much helpful advice throughout my three years at Cornell. I likewise thank my academic advisor and thesis group leader Dr. Ellis Loew, for his recommendations about undergraduate research and preparing for graduate school. My appreciation also extends to Colleen Kearns and Janet Snoyer, for listening, and for their cheerful encouragement.

I am sincerely thankful to all these individuals and countless others who have been a positive influence throughout my time at Cornell.

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Appendix 1: Supplementary Results

Table S1. Supplementary Results: Early Lipofectamine LTX & PLUS Reagent and XtremeGene HP Optimization.

The average fluorescence reading \pm standard deviation are provided. Note that this is not an actual transfection percentage, but is correlated with the actual transfection percentage.

For XtremeGene, three wells were used for each ratio/volume combination; for Lipofectamine, 18 wells were used for each ratio/volume combination. Because the gain setting on the plate reader was very low, the XtremeGene results are included as a point of reference.

It appears that lower volumes of Lipofectamine LTX & PLUS should be tested in the future, for they resulted in the highest fluorescence levels for that reagent. Two other interesting points to note are that, firstly, in this very early experiment, XtremeGene still produced higher fluorescence readings than FuGene (FuGene data not shown); also, the highest fluorescence readings within XtremeGene were obtained in the 2:1 and 1:1 ratios, which were later confirmed to be the most effective.

Fluorescence readings greater than 15 fluorescence units are bolded. The leftmost vertical column indicates the plating volume per well, in microliters; the ratios across the top indicate the reagent-to-DNA ratio.

XtremeGene HP

Cells-only control=1.06 \pm 0.32; XtremeGene-only control=0.87 \pm 0.33; DNA-only control=0.96 \pm 0.24

Ratio →	1:1	2:1	3:1	4:1
Volume ↓				
2	0.89 \pm 0.19	2.33 \pm 0.51	4.48 \pm 1.39	6.56 \pm 2.12
5	2.78 \pm 2.32	13.52 \pm 1.41	17.93\pm5.93	21.67 \pm 7.28
10	4.96 \pm 2.92	19.78\pm10.77	14.37 \pm 3.41	10.00 \pm 3.76
15	9.48 \pm 0.84	21\pm1.37	18.48\pm1.68	10.07 \pm 2.00
20	18.56\pm8.10	20.59\pm5.50	18.82\pm6.91	5.67 \pm 2.17
25	36.15\pm9.02	19.44\pm5.40	13.30 \pm 8.02	7.5926 \pm 1.72

Lipofectamine LTX & PLUS

Cells-only control=0.74 \pm 0.26; Reagent-only control=0.78 \pm 0.20; DNA-only control=0.76 \pm 0.18

Ratio →	1:1	2:1	3:1	4:1	5:1	6:1
Volume ↓						
10	5.19 \pm 3.60	13.70\pm7.50	17.48\pm4.94	31.04\pm11.18	20.85\pm6.14	14.78 \pm 12.81
20	7.30 \pm 4.09	1.63 \pm 0.57	5.11 \pm 6.26	10.41 \pm 5.17	8.63 \pm 4.06	7.07 \pm 3.22
30	1.41 \pm 0.17	3.11 \pm 0.87	2.85 \pm 0.42	2.96 \pm 0.84	0.85 \pm 0.26	1.22 \pm 0.59
40	2.22 \pm 1.94	1.37 \pm 0.74	1.70 \pm 0.95	1.59 \pm 0.95	1.44 \pm 0.84	0.85 \pm 0.17

Table S2. Supplementary Results: Failure of Expired XtremeGene to Elicit Typical XtremeGene Transfection Efficiency. Volume given is the μl added per well of a 96-well plate, with each well initially containing 100 μl of media. As demonstrated through the previous experiments, regular XtremeGene results in higher fluorescence than FuGene. But here, when expired XtremeGene was used, FuGene provided better transfection. Visual observation of transfected cells also confirmed the failure of expired XtremeGene to function as effectively as usual. Twenty-one wells were used for each particular ratio/volume combination in the table above.

Reagent	Ratio (μl of reagent to μg of DNA)	Volume	Fluorescence Reading (Average \pm St. Dev)
XtremeGene HP	1:2	28	3.35 ± 1.01
		32	3.73 ± 1.58
		36	3.49 ± 1.41
	1:1	32	149.04 ± 22.78
		36	108.44 ± 24.82
		40	77.97 ± 13.50
	2:1	24	81.62 ± 23.18
		28	49.90 ± 14.12
		32	44.74 ± 9.40
	3:1	8	47.88 ± 42.53
		12	92.62 ± 45.15
		16	65.93 ± 14.66
FuGene HD	5:2	22.5	204.08 ± 35.36
Controls	(reagent only, DNA only, cells only)		4.25 ± 1.33

Appendix 2: Solution Formulations

10% or 20% Fetal Bovine Serum Mesenchymal Stem Cell Media (with or without antibiotics)
(Referred to as “FBS MSC media” in the text; unless specifically noted in the text, antibiotics are included in the media)

Add the following to a 500mL bottle of Low Glucose DMEM (HyClone ThermoScientific, Logan, UT):

- 12.5 mL HEPES buffer (1 M, 238.3 mg/ml, Cellgro Mediatech Inc., Manassas, VA)
- 50 mL fetal bovine serum (HyClone FBS, Characterized; Canadian sourced. Cat. No. SH30396.03)
- 5 mL Pen/Strep (10,000 IU/mL Pen, 10,000 µg/mL Strep, Cellgro Mediatech Inc., Manassas, VA. Cat. No. 30-002-CI)
- 25 µl bFGF (100 µg/mL stock suspended in stem cell isolation media; Millipore, Danvers, MA)

Sterile filter into autoclaved bottle with 0.2 µM bottle-top filter. Store at 4°C.

For 20% FBS MSC media, add 100 mL FBS instead of only 50 mL FBS.

For antibiotic-free media, exclude Pen/Strep.

Mesenchymal Stem Cell Freeze Media

Combine the following in a glass bottle:

- 80mL stem cell isolation media
- 10 mL fetal bovine serum
- 10mL dimethyl sulfoxide (DMSO)

Sterile filter into an autoclaved bottle with 0.2 µM bottle-top filter. Do not add ingredients through the filter because DMSO will dissolve the filter membrane if added directly to the bottle-top filter apparatus. Store at -8°C.

FACS Sorting Buffer (with 1 mM EDTA and 1% FBS; optional DAPI)

Add the following to a 500 mL bottle of 1x PBS (Ca/Mg⁺⁺ free)

- 12.8 mL HEPES buffer (1 molar)
- 0.195 grams EDTA (tetrasodium salt, MW = 380.16)
- 5.2 mL fetal bovine serum (Ca/Mg⁺⁺-free if possible)
- 5 mL Pen/Strep
- Optional: add DAPI to obtain a concentration of 1 mg DAPI per 100 mL of sorting buffer. Solution should be stirred at 4°C overnight to increase dissolution of the DAPI.

Filter sterilize; store at 4°C

Appendix 3: Additional Protocols—MSC Isolation

MSC Isolation (24-hour Split Method)

This method provides somewhat higher cell numbers at first passage on average than other methods but is more labor-intensive.

Day 0

- 1) Pre-warm MSC isolation media in 37°C waterbath. For clinical cases, use 20% Serum MSC isolation media. For research cases, it is generally preferred to use the standard 10% serum.
- 2) Label one flask for every 20 mL of marrow, and add 30 mL of media to each flask. Ideally, each flask should be labeled to indicate which syringe was its source.
- 3) Clean syringes of marrow with ethanol before bringing into hood.
- 4) Add 20 mL of marrow to each flask.
- 5) Tilt flasks to make sure entire bottom surface is covered with liquid, being careful not to get liquid into the cap.
- 6) Place flasks into incubator overnight.

Day 1

- 7) Pre-warm appropriate MSC isolation media in 37°C waterbath
- 8) While media is warming, prepare additional flasks equal to the original number of flasks
- 9) Add 30 mL of isolation media to the original flasks, rinsing the culture surface as you add media
- 10) Remove 30-40 mL of the marrow-media mixture from each original flask and add it to a correspondingly labeled new flask
- 11) Tilt new flasks to make sure entire bottom surface is covered with liquid, being careful not to get liquid into the cap
- 12) Place all flasks into incubator at least overnight but for no longer than 48 hours

Day 2 or 3

- 13) Pre-warm appropriate MSC isolation media in 37°C water bath.
- 14) Aspirate media
- 15) Rinse flasks with 10 mL each of HBSS
- 16) Aspirate HBSS
- 17) Add 30 mL MSC isolation media

Ongoing

- 18) Repeat HBSS rinse 48 hours later if significant blood remains in flask, otherwise simply aspirate and feed. For clinical cases, once most blood is removed from plates, it is fine to switch to 10% serum standard MSC isolation media
- 19) Proceed to aspirate media and feed fresh media every 48 hours until moderately dense MSC colonies have formed on the culture surface—typically 5 to 11 days from initial plating depending on horse and quality of draw.
- 20) Assess number and density of colonies to determine the ideal surface area needed for first passage and prepare an appropriate number of plates
- 21) Proceed to stem cell passaging protocol

MSC Isolation (No Split Method)

This method is used when it is difficult or impossible for someone to work with the cells 24 hours after initial plating.

Day 0

- 1) Pre-warm MSC isolation media in 37°C water bath. For clinical cases, use 20% Serum MSC isolation media. For research cases, it is generally preferred to use standard 10% serum.
- 2) Label one flask for every 10 mL of marrow, and add 30 mL of media to each flask. Ideally, each flask should be labeled to indicate which syringe was its source.
- 3) Clean syringes of marrow with ethanol before bringing into hood
- 4) Add 10 mL of marrow to each flask
- 5) Tilt flasks to make sure entire bottom surface is covered with liquid, being careful not to get liquid into the cap
- 6) Place flasks into incubator at least overnight but for no longer than 48 hours

Day 1

- 7) Pre-warm appropriate MSC isolation media in 37°C water bath
- 8) Aspirate media
- 9) Add 30 mL MSC isolation media
- 10) Place flasks into incubator at least overnight but for no longer than 48 hours

Day 2 or 3

- 11) Pre-warm appropriate MSC isolation media in 37°C water bath
- 12) Rinse flasks with 10mL each of HBSS
- 13) Aspirate HBSS
- 14) Add 30 mL MSC isolation media

Ongoing

- 15) Repeat HBSS rinse 48 hours later if significant blood remains in flask. Otherwise simply aspirate and feed. For clinical cases, once most blood is removed from plates, it is fine to switch to 10% serum standard MSC isolation media
- 16) Proceed to aspirate media and feed fresh media every 48 hours until moderately dense MSC colonies have formed on the culture surface, typically 5 to 11 days from initial plating depending on horse and quality of draw
- 17) Assess number and density of colonies to determine the idea surface area needed for first passage and prepare an appropriate number of plates
- 18) Proceed to stem cell passaging protocol